**DEPARTAMENTO DE BIOLOGÍA CELULAR,
FISIOLOGÍA E INMUNOLOGÍA**

Identificación de nuevos marcadores moleculares y/o celulares implicados en la respuesta farmacológica y contribución de mecanismos de splicing en los tumores hipofisarios

Identification of novel cellular and/or molecular markers involved in the pharmacological response and contributions of splicing mechanisms in pituitary tumors

Memoria de Tesis Doctoral presentada por **María del Carmen Vázquez Borrego**, Licenciada en Biología y Bioquímica, para optar al grado de **Doctora en Biomedicina**

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INFORMAN

Que Dª María del Carmen Vázquez Borrego, Licenciada en Biología y Bioquímica, ha realizado bajo nuestra dirección el trabajo titulado **"Identification of novel cellular and/or molecular markers involved in the pharmacological response and contributions of splicing mechanisms in pituitary tumors"** y que bajo nuestro juicio reúne los méritos suficientes para optar al Grado de Doctora en Biomedicina.

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TÍTULO DE LA TESIS: ***Identification of novel cellular and/or molecular markers involved in the pharmacological response and contributions of splicing mechanisms in pituitary tumors***

DOCTORANDO/A: María del Carmen Vázquez Borrego

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Durante el desarrollo de la presente Tesis Doctoral, en el periodo comprendido entre noviembre de 2014 y junio de 2019, la doctoranda María del Carmen Vázquez Borrego ha alcanzado e incluso superado con creces los objetivos planteados al comienzo de la misma, al tiempo que ha desarrollado técnicas experimentales de gran utilidad para el grupo de investigación, que le han permitido obtener resultados muy relevantes en el campo de los tumores hipofisarios y que quedan patentes en varias publicaciones. Concretamente, como fruto de su trabajo durante este periodo, ha publicado tres trabajos directamente relacionados con su Tesis Doctoral, en las revistas *"Cellular Physiology and Biochemistry"*, *"The Journal of Clinical Endocrinology and Metabolism"* y *"Neuroendocrinology"*, revistas de referencia dentro de nuestras áreas de investigación. Además, el trabajo realizado en este periodo ha dado lugar a otros tres artículos, los cuales están sometidos o a punto de ser sometidos a las revistas *"Acta Neuropathologica", "The Journal of Clinical Endocrinology and Metabolism" y "EBiomedicine".*

Por último, la doctoranda ha presentado sus resultados en diferentes congresos de ámbito nacional e internacional, de los que han derivado varios capítulos de libro. Además, como resultado de sus trabajos, la doctoranda ha recibido varios premios procedentes de la "Sociedad Española de Endocrinología y Nutrición", "Sociedad Andaluza de Endocrinología y Nutrición" y de las "9ª Jornadas de Jóvenes Investigadores del IMIBIC".

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 14 de mayo de 2019

Firma de los directores

Fdo.: Raúl M. Luque Huertas

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Esta Tesis Doctoral ha sido realizada en el Departamento de Biología Celular, Fisiología e Inmunología de la Universidad de Córdoba, bajo la dirección de los Dres. Raúl M. Luque Huertas y Justo P. Castaño Fuentes. Dicho proyecto fue subvencionado mediante proyectos de la Junta de Andalucía (CTS-1406 and BIO-0139), del Instituto de Salud Carlos III (PI13/00651, PI16/00264), del Ministerio de Ciencia, Innovación y Universidades (BFU2016-80360-R) y del Centro de Investigación Biomédica en Red de la Fisiopatología de la Obesidad y Nutrición (CIBERobn).

“Me lo contaron y lo olvidé;
lo vi y lo entendí;
lo hice y lo aprendí”

Confucio

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A Víctor, mis padres y mis hermanos

List of Abbreviations

GH – Growth hormone

ACTH – corticotropin

PRL – prolactin

FSH – follicle-stimulating hormone

LH – luteinizing hormone

TSH – thyrotropin

GHRH – growth-hormone releasing hormone

GNRH – gonadotropin releasing hormone

CRH – corticotropin releasing hormone

TRH – thyrotropin releasing hormone

SRIF – somatostatin

DA – dopamine

PACAP – pituitary adenylate cyclase activating polypeptide

CNS – central nervous system

SST – somatostatin receptor

TMD – transmembrane domain

SSA – somatostatin analogue

PitNET – pituitary neuroendocrine tumor

cAMP – cyclic adenosine monophosphate

PKA – activate cAMP-dependent protein kinase

FFA – free fatty acid

IGF1 – insulin-like growth factor 1

snRNA – small nuclear RNA

snRNP – ribonucleoprotein

MRI – magnetic resonance imaging

Non-funtioning pituitary tumor – NFPT

FSH/LHoma – gonadotropinoma

PRLoma – prolactinoma

GHoma – somatotropinoma

ACTHoma – corticotropinoma

TSHoma – thyrotropinoma

FPT – functioning pituitary tumor

NP – normal pituitary

MF – metformin

BF – buformin

PF – phenformin

T2DM – type-2 diabetes mellitus

NET – neuroendocrine tumor

AICAR – AMP mimetic compound 5-aminoimidazole-4-carboxamide ribonucleoside

AMPK – AMP-activated protein kinase

AC – adenylate cyclase

PLC – phospholipase C

MAPK – mitogen-activated protein kinase

PKC – protein kinase C

GC – guanylate cyclase

BMI – body mass index

CgA – chromogranin A

PLS-DA – Partial Least Squares-Discriminant

SF – splicing factor

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Resumen

1. Resumen

La **hipófisis** es una glándula endocrina de pequeño tamaño pero de vital importancia localizada en la base del cerebro y constituida por dos regiones, una glandular, la adenohipófisis, compuesta por los lóbulos anterior e intermedio, y otra de origen neural, la neurohipófisis o lóbulo posterior. La **adenohipófisis** está compuesta por cinco tipos de células endocrinas productoras de hormonas entre las que se encuentran las células somatotropas, lactotropas, corticotropas, gonadotropas y tirotropas, las cuales sintetizan y secretan hormona del crecimiento (GH), prolactina (PRL), corticotropina (ACTH), hormona foliculoestimulante (FSH) y luteinizante (LH), y tirotropina (TSH), respectivamente. La hipófisis es responsable del control de múltiples funciones biológicas (crecimiento, reproducción, metabolismo, respuesta al estrés, etc.) y está regulada por una compleja red de señales moduladoras que actúan a través de diversos receptores para integrar y procesar toda la información recibida con el fin de controlar la síntesis y secreción de las diferentes hormonas hipofisarias. Específicamente, esta regulación fina es ejercida por señales centrales (GHRH, GNRH, CRH, TRH, somatostatina, dopamina, etc.) y periféricas (glucocorticoides, adipoquinas, hormonas tiroideas, testosterona, estrógenos, ghrelina, obestatina, etc.) y el adecuado balance entre todas estas señales aseguran el apropiado funcionamiento de la glándula y, por lo tanto, del organismo.

La alteración neoplásica de algunos de los tipos celulares hipofisarios da lugar al desarrollo de los **tumores neuroendocrinos hipofisarios**, los cuales constituyen el 15% de todos los tumores cerebrales. Diversos estudios han descrito que estos tumores se generan a partir de una expansión monoclonal de células alteradas genéticamente y que la desregulación de los factores reguladores mencionados previamente tendría un papel importante en dicha transformación. Desde un punto de vista clínico, los tumores hipofisarios se clasifican en tumores hipofisarios no funcionantes (NFPTs), gonadotropinomas (FSH/LHomas), prolactinomas (PRLomas), somatotropinomas (GHomas), corticotropinomas (ACTHomas) y tirotropinomas (TSHomas). Aunque la cirugía trans-esfenoidal es la primera opción de terapia para pacientes con tumores hipofisarios, en muchos casos no se consigue un control adecuado de la enfermedad y es necesario el uso de terapias farmacológicas. En este sentido, los análogos de somatostatina (octreótido, pasireótido y lanreótido) y los agonistas de dopamina (cabergolina) son las opciones terapéuticas que se emplean actualmente en la práctica clínica. Sin embargo, aunque estos fármacos han demostrado tener una alta eficacia reduciendo el tamaño tumoral y la hipersecreción hormonal, distintos estudios demuestran que muchos pacientes son (o se vuelven) resistentes a estos tratamientos. Por ello, es necesaria la

búsqueda de nuevas opciones terapéuticas que enriquezcan el conjunto de fármacos actualmente disponibles para tratar estas patologías tumorales.

En este sentido, una de las estrategias actuales para la generación de nuevos fármacos es la síntesis de derivados químicos de drogas actuales (análogos de somatostatina, agonistas de dopamina) que presenten mayor afinidad, potencia y/o eficacia que los actuales. Adicionalmente, una fuente para la identificación de nuevos fármacos es el reposicionamiento de drogas que están actualmente en el mercado y que son clínicamente seguras, como el caso de las biguanidas (como la metformina) y las estatinas, ya que son tratamientos farmacológicos que se emplean actualmente en la práctica clínica para controlar alteraciones metabólicas causadas por condiciones patológicas como las diabetes, la obesidad o la hipercolesterolemia y que podrían ejercer acciones directas a nivel de la glándula hipofisaria modulando su función bajo condiciones normales y/o patológicas. Finalmente, cabe también destacar que aunque la causa inicial de la aparición de los tumores neuroendocrinos hipofisarios aún no se ha esclarecido totalmente, estudios recientes sugieren que la alteración de los procesos fisiológicos de splicing alternativo y la aparición de variantes aberrantes de splicing es una característica común en la mayoría de las patologías tumorales y, por lo tanto, podría representar una nueva vía para la identificación de nuevas dianas para el diagnóstico, pronostico y/o tratamiento de las patologías tumorales.

Por todo esto, el **objetivo principal** de esta Tesis ha sido profundizar en un conocimiento celular, molecular y clínicamente relevante de la regulación fisiopatológica de la glándula hipofisaria, y de los tumores neuroendocrinos hipofisarios, a través de la identificación de nuevos factores y mecanismos involucrados en la respuesta funcional a diferentes terapias farmacológicas.

En primer lugar, los resultados obtenidos en esta Tesis Doctoral demuestran que las biguanidas (metformina, buformina y fenformina), familia de compuestos antidiabéticos, ejercen efectos directos en células derivadas de diferentes tipos de tumores hipofisarios, reduciendo la proliferación celular y la secreción hormonal, e incrementando la apoptosis en GHomas, al menos en el caso de la metformina. Todos estos efectos parecen estar mediados por la modulación de mecanismos dependientes ($[Ca^{2+}]_i$ y ruta PI3K) e independientes de AMPK (ruta de ERK). Además, evaluamos la posible asociación entre el tratamiento con metformina y diversos parámetros clínicos en pacientes con tumores hipofisarios. Sin embargo, el uso de metformina no se relacionó con ninguna de las variables clínicas

determinadas en nuestra cohorte de pacientes, lo cual podría ser debido al limitado número de pacientes tratados con este fármaco. De forma paralela, encontramos que la metformina puede afectar directamente a las células somatotropas, corticotropas y gonadotropas normales de dos especies de primates (*Papio anubis* y *Macaca fascicularis*), alterando la secreción hormonal, la expresión génica y la señalización celular, todo ello sin afectar a la viabilidad celular. Así, el efecto de la metformina sobre la secreción de GH, ACTH y FSH se produjo a través de las rutas de mTOR, PI3K y de la movilización de Ca^{2+} intracelular. Además, la ruta de la MAPK también fue esencial para las acciones de la metformina sobre la secreción de GH. Estos resultados podrían tener una gran relevancia translacional puesto que las acciones inhibidoras de la metformina sobre la secreción de GH y ACTH podrían ser beneficiosas desde el punto de vista metabólico ya que varios estudios han demostrado que el incremento de los niveles circulantes de GH y de glucocorticoides puede desencadenar resistencia a la insulina, intolerancia a la glucosa y diabetes mellitus.

En la misma línea, durante el desarrollo de esta Tesis también se han estudiado los efectos directos de la simvastatina (compuesto de la familia de las estatinas usado en clínica para reducir la hipercolesterolemia) sobre parámetros funcionales relevantes en cultivos primarios de diferentes tipos de tumores hipofisarios y líneas celulares, así como su efecto en cultivos primarios de células hipofisarias normales procedentes de una especie de primate (*Papio anubis*). Nuestros resultados demuestran que la simvastatina puede reducir la viabilidad celular y la secreción hormonal en células de tumores hipofisarios a través de la modulación de MAPK. Sin embargo, la combinación de simvastatina con metformina o análogos de somatostatina no resultó en ningún efecto aditivo de estos fármacos. Al igual que en el caso de la metformina, exploramos la posible asociación entre el tratamiento con estatinas y diversos parámetros clínicos en pacientes con tumores hipofisarios. En este caso, los resultados revelaron que los pacientes tratados con estatinas mostraron una tendencia a un menor crecimiento extraselar que los pacientes no tratados. En cambio, ningún otro parámetro se relacionó con el uso de estatinas. En relación con las células hipofisarias normales, la simvastatina redujo la secreción de ACTH, GH, PRL, FSH y LH, sin alterar la expresión génica o la viabilidad celular. Estos efectos fueron mediados a través de mTOR y PI3K, y también a través de MAPK en el caso de los efectos sobre ACTH, GH y PRL.

Por otro lado, en esta Tesis hemos identificado y caracterizado, por primera vez, el efecto de dos agonistas específicos del receptor de somatostatina tipo 3 (SST_3) sobre parámetros funcionales clave en cultivos primarios de NFPTs. Así, nuestros resultados demuestran que

estos compuestos pueden reducir la viabilidad celular, la secreción de cromogranina-A e incrementar la apoptosis, siendo BIM-355 el compuesto más potente. Además, BIM-355 también redujo el crecimiento tumoral en un modelo preclínico de tumor hipofisario de ratón. El estudio de diferentes rutas de señalización reveló que BIM-355 ejerce sus efectos a través de la modulación de las rutas MAPK, PI3K-Akt/mTOR y JAK/STAT. Curiosamente, encontramos una proporción de NFPTs no respondedores a estos fármacos, en los cuales sólo la expresión de SST_3 (a nivel de ARNm y de proteína), pero no de otros receptores de somatostatina, fue capaz de discriminar entre las dos poblaciones (respondedores y no respondedores). Así, este estudio proporciona un conjunto sólido de pruebas que demuestran que el SST_3 tiene un papel funcional y relevante y con potencial terapéutico en la fisiopatología de los NFPTs.

Otra aproximación realizada en esta Tesis ha sido el estudio de los efectos directos de un nuevo compuesto quimérico, conocido como BIM-065 (nueva generación de dopastatina), capaz de unirse a los receptores de somatostatina tipo 2 (SST_2) y 5 (SST_5) y al receptor de dopamina tipo 2 (D_2) sobre diferentes tipos de tumores hipofisarios y líneas celulares. En este sentido, BIM-065 redujo la viabilidad celular y la secreción hormonal e incrementó la apoptosis en diferentes tipos de tumores hipofisarios. Además, el estudio de diferentes rutas de señalización en la línea celular AtT-20 reveló que una incubación breve con BIM-065 (10 min) aumenta los niveles de fosforilación de Akt, lo cual fue seguido de una clara reducción de la proteína antiapoptótica Bcl-2 (lo que apoya el incremento de apoptosis observado en respuesta a BIM-065). En cambio, una incubación a largo plazo (24h) con BIM-065 provocó un aumento en los niveles de fosforilación de ERK1/2. De acuerdo con los resultados descritos en la literatura, estos resultados podrían sugerir que este compuesto quimérico modula ERK1/2 y Akt a través de la activación preferencial de la señalización dopaminérgica. Más importante aún, y en contraste con datos previos de otros compuestos quiméricos, el tratamiento con BIM-065 no generó ningún efecto estimulador en las células tumorales analizadas.

Finalmente, nuestros resultados también indican que la maquinaria celular responsable del procesamiento y la regulación del proceso de *splicing* (spliceosoma y factores de *splicing*) se encuentra diferencialmente desregulada en diferentes tipos de tumores hipofisarios en comparación con hipófisis normal. Estos resultados también revelaron una huella específica de componentes desregulados de la maquinaria de splicing capaz de discriminar entre cada tipo tumoral y el tejido hipofisario sano. Además, se encontraron varios componentes desregulados (SRSF1, RNU11, RNU4ATAC y RNU6ATAC) de forma común en todos los tumores hipofisarios analizados, lo cual podría sugerir la existencia de alteraciones comunes en la

maquinaria de splicing que podrían estar implicadas en la tumorigenesis de estas patologías y, de hecho, abre el camino hacia la identificación de nuevas dianas terapéuticas comunes basadas en la desregulación de estos elementos. Más aún, nuestro estudio también demuestra que la alteración farmacológica del *splicing* con fármacos específicos, como el pladienolide-B (compuesto dirigido contra SF3B1, un componente clave involucrado en el ensamblaje del spliceosoma), es capaz de reducir la viabilidad celular y la secreción hormonal en células de tumores hipofisarios.

En resumen, los resultados de esta Tesis proporcionan una información novedosa y relevante acerca de los efectos antitumorales que ejercen sobre diferentes tipos de tumores neuroendocrinos hipofisarios distintos fármacos, algunos de ellos utilizados en la práctica clínica habitual con otras indicaciones, y otros que son nuevos fármacos dirigidos contra receptores de somatostatina/dopamina, y que, en conjunto, sugieren que estos compuestos podrían llegar a ser opciones terapéuticas prometedoras en el tratamiento de pacientes con tumores neuroendocrinos hipofisarios. Además, esta Tesis demuestra que la alteración específica de la maquinaria de *splicing* podría estar involucrada en la génesis de los tumores hipofisarios y podría proporcionar nuevas herramientas para mejorar el diagnóstico, pronóstico y las opciones terapéuticas para estas patologías.

Summary

2. Summary

The **pituitary gland** is a small but functionally crucial endocrine organ localized at the base of the brain and is comprised by two distinct regions, one of glandular nature, the adenohypophysis, comprised by the anterior and intermediate lobes, and another of neural origin, the neurohypophysis or posterior lobe. The **adenohypophysis** is mainly composed by five hormone-producing endocrine cells including somatotropes, lactotropes, corticotropes, gonadotropes and thyrotropes, which are responsible for the synthesis and secretion of growth hormone (GH), prolactin (PRL), corticotropin (ACTH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and thyrotropin (TSH), respectively. The pituitary gland is responsible for the control of multiple biological functions (i.e. growth, reproduction, metabolism, stress response, etc.) and is modulated by a complex network of regulatory signals that act through different cellular receptors to integrate and process the information at the intracellular signal transduction level to finely control synthesis and secretion of the different anterior pituitary hormones. This fine regulation is exerted by central (GHRH, GNRH, CRH, TRH, somatostatin, dopamine, etc.) and peripheral signals (glucocorticoids, adipokines, thyroid hormones, testosterone, estrogens, ghrelin, obestatin, etc.) and the adequate balance among all these regulatory signals ensure the appropriate function of the pituitary gland and, therefore, of the whole organism.

The neoplastic alteration of these pituitary cells may lead to the development of pituitary neuroendocrine tumors (PitNETs), which represent 15% of all brain tumors. Several studies have reported that these tumors arise from a monoclonal expansion of genetically altered cells and that the dysregulation of regulatory central and peripheral factors mentioned above may play a crucial role in this transformation. From a clinical point of view, PitNETs are classified in non-functioning pituitary tumors (NFPTs), gonadotropinomas (FSH/LHomas), prolactinomas (PRLomas), somatotropinomas (GHomas), corticotropinomas (ACTHomas) and thyrotropinomas (TSHomas). To date, transsesphenoidal surgery is considered the first-line therapeutic option for patients harboring PitNETs; however, in many cases, there is an inadequate disease control and it is necessary to use subsequent pharmacological therapy. Particularly, somatostatin analogues (octreotide, pasireotide and lanreotide) and dopamine agonists (cabergoline) are the main therapeutic medical options currently available. However, although these drugs have demonstrated a great efficacy inducing tumor shrinkage and reducing hormone hypersecretion, several studies have shown that some patients are (or become) unresponsive to these treatments. For this reason, the search for new therapeutic options to enrich the pharmacological arsenal to treat PitNETs patients is urgently necessary.

In this sense, one of the current strategies to the generation of novel therapeutic compounds is the synthesis of chemical derivatives of current drugs (somatostatin analogues and dopamine agonists) that could exhibit higher affinity, potency and/or efficacy. An additional source for the identification of novel therapeutic compounds is the repositioning of currently available drugs that have been proved to be clinically safe, as it is the case of biguanides (as metformin) or statins. Indeed, they are pharmacological treatments currently used in the clinical practice to control metabolic alterations due to pathological conditions such as diabetes, obesity or hypercholesterolemia, and could therefore also exert some actions at the pituitary gland and modulate its function under normal and/or pathological conditions. Finally, it should be also noted that although the primary initiating cause of PitNETs development is still unclear, recent studies suggest that altered alternative splicing and appearance of aberrant splicing variants is a common feature of most tumor pathologies and could, therefore, represent a novel avenue for the identification of novel targets for the diagnostic, prognostic and treatment of PitNETs.

Thence, general **aim of this study** was to gain deeper cellular, molecular and clinically-relevant knowledge on the (patho)physiological regulation of the pituitary gland, and of PitNETs, through the identification of novel factors and mechanisms involved in the functional response to different pharmacological therapies, including hormone release and the development and progression of pituitary tumor pathology.

Firstly, the findings presented herein demonstrate that biguanides (metformin, buformin and phenformin) exert direct antiproliferative and antisecretory actions in different PitNETs cell types, and that metformin also increases apoptosis in GH-secreting PitNETs. All these actions were mediated through the modulation of AMPK-dependent ($[Ca^{2+}]_i$ kinetics and PI3K-Akt pathway) and independent (ERK pathway) mechanisms. Moreover, we evaluated the putative association between treatment with metformin and clinical parameters in patients harboring different PitNETs subtypes. However, metformin use was not apparently related to any clinical variable determined in our cohort of patients, which could be due to the limited number of patients treated with metformin. In parallel, we also found that metformin impacts directly on pituitary somatotrope, corticotrope and gonadotrope cells at the secretory, gene expression and signaling levels, without altering cell viability, in normal pituitary cells from two non-human primate species (*Papio anubis* and *Macaca fascicularis*). In particular, we found that the effect of metformin on GH, ACTH and FSH release were mediated through mTOR, PI3K

and intracellular Ca^{2+} mobilization. Additionally, the actions of metformin on GH secretion also involved MAPK pathway. Interestingly, these findings could have translational relevance in that the decrease on GH and ACTH secretion could be a beneficial metabolic action of MF to improve whole body homeostasis, inasmuch as several studies have reported that increased circulating levels of GH and glucocorticoids can lead to worsening of insulin resistance, glucose intolerance and diabetes mellitus.

We also explored during this Thesis the direct effects of simvastatin on functional parameters in primary cell cultures from different PitNETs subtypes and cell lines, as well as in primary normal pituitary cell cultures from *Papio anubis*. Our results demonstrated that simvastatin reduced cell viability and hormone secretion in PitNETs cells through the modulation of MAPK signaling pathway. Remarkably, combination therapy of simvastatin with metformin or SSAs did not show additive effects in AtT-20 and GH3 cells, suggesting shared mechanisms of action between these drugs. Moreover, we also explored the putative association between treatment with statins and clinical parameters of patients harboring different PitNETs subtypes. In this case, the analysis revealed that patients treated with statins showed a trend to have less extrasellar growth compared with patients not treated with statins. None of the other clinical parameters evaluated herein revealed significant associations with the use of statins. Regarding normal pituitary cells, simvastatin also reduced ACTH, GH, PRL, FSH and LH secretion, without altering hormone gene expression or cell viability. In this case, these effects were mediated trough mTOR and PI3K pathways, and the actions on ACTH, GH and PRL secretion also required MAPK pathway.

On the other hand, we identified and characterized, for the first time, the effect of two somatostatin receptor type 3 (SST_3)-specific agonists on key functional parameters in primary cell cultures from NFPTs, where this receptor is overexpressed compared with other somatostatin receptors subtypes. Our results showed that these compounds reduced cell viability and chromogranin-A secretion, and increased apoptosis, being BIM-355 the most potent compound. Furthermore, BIM-355 also reduced tumor growth in a preclinical mouse model of PitNET. The study of different signaling pathways revealed that BIM-355 exerted its actions through the modulation of MAPK, PI3K-Akt/mTOR and JAK/STAT pathways. Importantly, we found a proportion of NFPTs unresponsive to these drugs, which was associated to the presence of SST_3, in that only SST_3 expression (at mRNA and protein levels), but not the other SST-subtypes, was able to discriminate between responsive and

unresponsive tumors. Thus, this study provided compelling evidence demonstrating that SST_3 has a relevant functional role and therapeutic potential in the pathophysiology of NFPTs.

Additionally, we evaluated the direct effects of a new chimeric $SST_2/SST_5/D_2$ compound, called BIM-065 (new dopastatin class), in different PitNETs subtypes and pituitary cell lines. BIM-065 acted mostly reducing cell viability and hormone secretion and increasing apoptosis in the different PitNETs subtypes. The study of different signaling pathways in AtT-20 cells revealed that short-term incubation with BIM-065 (10 min) increased phospho-Akt, which was also followed by a clear reduction of anti-apoptotic protein Bcl-2 (supporting the increase of apoptosis observed in PitNETs cells). In contrast, long-term incubation (24h) upregulated phospho-ERK1/2 levels. Based on the literature, these results might suggest that BIM-065 modulate ERK1/2 and Akt through the preferential activation of the D_2/dopaminergic-signaling. Most importantly, in contrast to previous data with other chimeric compounds, treatment with BIM-065 did not evoke any stimulatory action in the tumor cells analyzed.

Finally, our results also indicate that the cellular machinery responsible for the processing and regulation of the splicing process (spliceosome and splicing factors) is differentially dysregulated in PitNETs compared to normal pituitary glands (NPs). These results also revealed a unique fingerprint of spliceosome components in each PitNET subtype that accurately discriminate between them and NPs. Furthermore, we also found several components (SRSF1, RNU11, RNU4ATAC and RNU6ATAC) that were commonly dysregulated in all PitNETs analyzed which may suggest the existence of common driver alterations in pituitary tumorigenesis and may pave the way toward the identification of common therapeutic targets based on the dysregulations of these key elements. Indeed, our study also demonstrated that the pharmacological disruption of the splicing process with specific drugs, such as pladienolide-B (compound able to directly target to SF3B1, a key component involved in the assembly of the spliceosome), produced the reduction of cell viability and hormone secretion in PitNETs cells.

In summary, the results of this Thesis provide novel and relevant information about the antitumor effects of different drugs currently used in clinical practice, as well as of new drugs targeting specific somatostatin/dopamine receptors, in different PitNETs, suggesting that these compounds could become a promising option to treat patients harboring PitNETs. In addition, this Thesis demonstrate that the alteration of the splicing machinery could be involved in the tumorigenesis of PitNETs and could also provide new tools to identify novel diagnostic, prognostic and potential therapeutic targets in this pathology.

Introduction

3. Introduction

3.1 The pituitary gland

The pituitary gland is a small endocrine organ localized at the base of the brain, in a depression of the sphenoid bone named the *sella turcica*. It is functionally and anatomically connected to the hypothalamus through the median eminence, and it is constituted by two distinct portions: a glandular component or **adenohypophysis** and a neural portion or **neurohypophysis.** The **adenohypophysis** comprises the **anterior lobe** (*pars distalis*), the intermediate lobe (*pars intermedia*; poorly developed in humans), and the *pars tuberalis***;** whereas, the **neurohypophysis** is constituted by the **posterior lobe** (*pars nervosa*) and the infundibulum [1, 2]**(Figure 1)**.

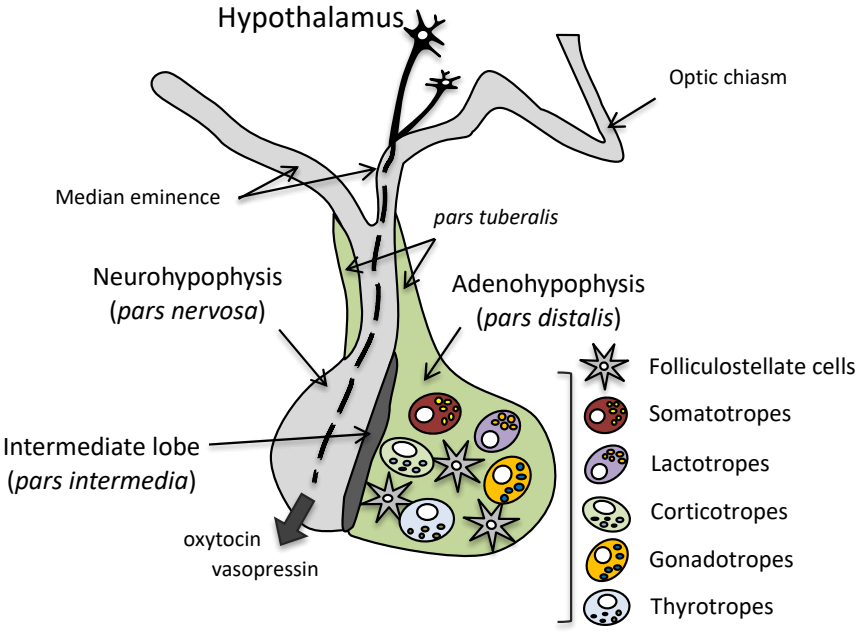

Figure 1: Pituitary gland anatomy. The pituitary is constituted by the neurohypophysis, which is composed by axonal terminals surrounded by pituicytes, and the adenohypophysis, which is composed by folliculostellate cells and five hormone-producing epithelial cell types. Adapted from [2].

The neurohypophysis is developed from the embryonic neuroectodermal layer and is constituted by axonal terminals of the hypothalamus supraoptic and paraventricular nuclei, which are surrounded by pituicytes (modified glial cells) that contribute to regulate neurohypophysial hormone release. Thus, oxytocin and vasopressin are hypothalamic hormones stored and released from this lobe to the systemic circulation [1]. In addition, hypothalamic factors reach the pituitary gland through hypophysial portal circulation to regulate adenohypophysial cells. These hypophysiotropic hormones comprise stimulatory

factors such as growth-hormone releasing hormone (GHRH), gonadotropin releasing hormone (GNRH), corticotropin releasing hormone (CRH) or thyrotropin releasing hormone (TRH), and inhibitory factors such as somatostatin (SRIF) or the monoamine dopamine (DA), which, together with peripheral signals derived from target organs, regulate the synthesis and secretion of the pituitary hormones [1-3].

The adenohypophysis is developed from the Rathke's pouch, which is derived from the roof of the stomodeum, and is considered the "master gland" of the endocrine system, as it is involved in the control of an ample range of critical physiological functions, including growth, puberty, reproduction, lactation, metabolism and stress, through the integration of multiple central, peripheral and intracellular signals [1, 4]. The anterior pituitary gland is composed by folliculostellate (FS) cells (non-endocrine cells; ~5-10% of total pituitary cells) and five hormone-producing endocrine cells including somatotropes (~40-50% of cell population), lactotropes (~15%), corticotropes (~15-20%), gonadotropes (~10%), and thyrotropes (~5%) **(Figure 1)**, which are responsible for the synthesis and secretion of growth hormone (GH; regulates bone and muscle growth and maintains lean body mass in adults), prolactin (PRL; stimulates breast milk production and regulates gonadal function), corticotropin (ACTH; stimulates glucocorticoids synthesis by the adrenal gland), follicle-stimulating hormone (FSH) and luteinizing hormone (LH; both FSH and LH regulate germ-cell development and sexual hormone synthesis/release by the gonads), and thyrotropin (TSH; stimulates thyroid hormones production by the thyroid gland), respectively [1, 3, 5]. On the other hand, FS cells are non-endocrine cells located in the parenchymal tissue of the adenohypophysis. These cells have a star-like morphology and produce many factors involved in the control of the behavior of surrounding cells and the gland itself. In addition, FS cells constitute an intra-pituitary regulatory deposit of cell residues due to their ability to effect phagocytosis [1, 6].

3.2 Important modulators of pituitary cell function.

The pituitary gland is finely modulated by a complex network of multiple regulatory signals that act through specific receptors to integrate and process the information at the intracellular signal transduction level to finely control synthesis and secretion of the different anterior pituitary hormones. The primary control of pituitary secretion is mainly exerted by the hypothalamus through the hypothalamic hormones. However, nowadays, it is well accepted that the precise regulation of the pituitary gland is exerted by both central (hypothalamic) and peripheral signals [4].

Regarding the central modulators, a plethora of stimulatory and inhibitory factors have been discovered. Classical stimulatory factors include GHRH, GNRH, CRH and TRH, which are responsible of GH, FSH/LH, ACTH and TSH secretion, respectively. TRH, and under some circumstances GHRH, also stimulate PRL [4]. In addition, other central stimulatory factors have been described such as ghrelin, which stimulates GH, PRL and ACTH release; pituitary adenylate cyclase activating polypeptide (PACAP), that stimulates GH, PRL and ACTH release; kisspeptin, that stimulates GH and FSH/LH; cortistatin, a neuropeptide that can stimulate GH and PRL release at low concentrations; and melatonin, produced by the pineal gland, which stimulates GH, PRL and FSH/LH release [4]. On the other hand, there are several important inhibitory factors, including the peptide somatostatin (SRIF), which primarily inhibits GH release from somatotropes but can also inhibit other pituitary hormones; and the monoamine dopamine (DA) which inhibits primarily PRL secretion but can also reduce GH and TSH release. In addition, there are other inhibitors such as neuropeptide Y, which can inhibit GH and FSH/LH release, and cortistatin, able to inhibit GH and also ACTH secretion [4].

Additionally, a high number of peripheral modulators (see section 1.2.3) [4] and environmental cues, like the circadian rhythm oscillations related to light/dark or sleep/wake cycles, have been also reported to regulate pituitary hormone secretion [7].

3.2.1 Somatostatin and somatostatin receptors.

Somatostatin, also known as somatotropin release-inhibiting factor (SRIF), is a peptide originally isolated in 1973 from ovine hypothalamus owing to its ability to inhibit GH secretion [8]. The *SRIF* gene is encoded on human chromosome 3 [9], and its transcribed mRNA is translated into a 116-amino acid precursor protein, pre-pro-somatostatin, that is processed to give rise to two main bioactive isoforms: the most abundant is the cyclic tetradecapeptide somatostatin-14 (SRIF-14), usually referred to as SRIF, whereas somatostatin-28 (SRIF-28) is an extended 28-amino acid peptide, also cyclic , and can also be processed to generate SRIF-14 **(Figure 2)** [10-13]. In addition, mammalian processing of pre-pro-somatostatin can give rise to another non-cyclic amidated peptide with 13-amino acid, named neuronostatin [14].

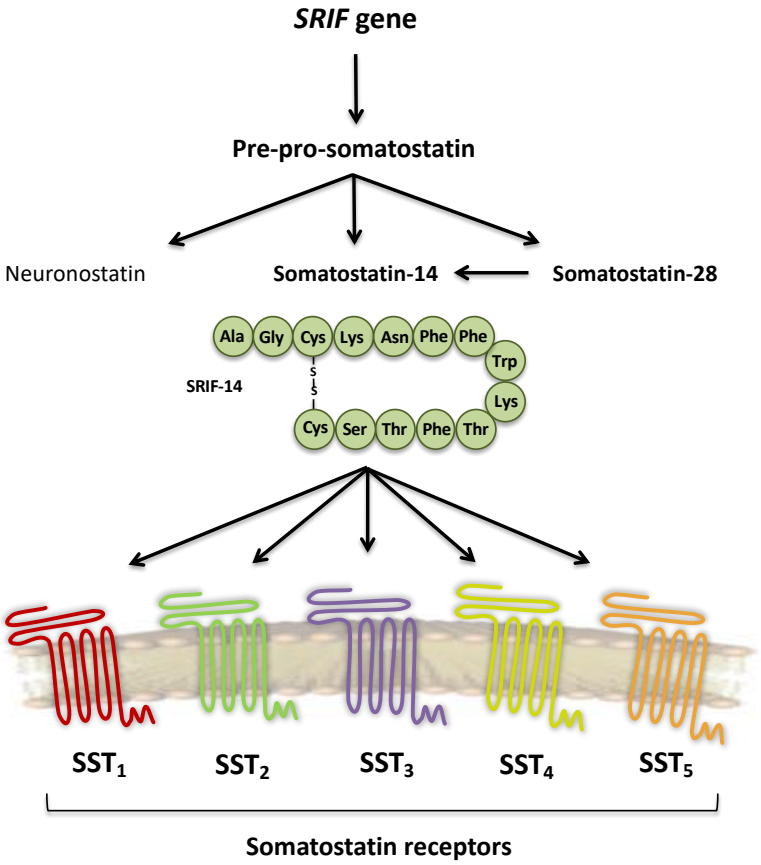

Figure 2: Somatostatin gene derived peptides including somatostatin-28, somatostatin-14 and neuronostatin. SRIF-14 exerts its actions through the binding to five classical somatostatin receptors.

SRIF is widely distributed throughout the central nervous system (CNS) and peripheral tissues, where it exerts numerous physiological actions including the modulation of cognitive functions through the regulation of neurotransmission, inhibition of pituitary hormone release, regulation of gastrointestinal tract by inhibiting endocrine and exocrine secretions, motility, blood flow, absorption and growth, inhibition of pancreatic enzymes and neuropeptides, and it is also able to inhibit cell proliferation of tumoral and normal cells [15-18].

SRIF exerts all its actions by binding to five different receptors, named SST_{1-5}, which belong to the seven transmembrane G protein-coupled receptor superfamily [10-13, 19] **(Figure 2)**. Taking into account that each SST subtype has been associated with the activation of specific signaling pathways to convey different SRIF functions, and that most tissues often co-express different SST subtypes, the precise action of SRIF is highly dependent on the precise amount of the different SSTs, the possible interaction between them, as well as the specific set of signaling pathways activated in response to SRIF [10-12, 19, 20].

In addition to the canonical, full length SST_{1-5}, two novel SST_5 truncated variants have been reported, which were named SST_5TMD4 and SST_5TMD5 due to the number of transmembrane domains (TMDs) [21]. To date, several reports have demonstrated that the

presence of these variants have functional consequences in different tissues [22-27]. Moreover, although the expression of both receptors is very low in normal tissues, SST_5TMD4 is highly frequent in pituitary neuroendocrine tumors (PitNETs) [21]. Thus, the presence of SST_5TMD4 has been related with sinus invasion, poor pharmacological response to somatostatin analogues (SSAs) and with an increase on the aggressiveness of GH-secreting PitNETs [23].

3.2.2 Dopamine and dopamine receptors.

Dopamine (DA) is a catecholamine neurotransmitter widely distributed in the mammalian brain. DA is synthetized from the aromatic amino acid tyrosine, and is involved in an ample range of central and peripheral functions including cognition, emotion, food intake, locomotor activity, positive reinforcement, hormone secretion, cardiovascular regulation, vascular tone, renal function, and gastrointestinal motility [28, 29].

DA exerts its functions through binding to five different receptors, named $D_{1\text{-}5}$, which belong to the seven transmembrane G protein-coupled receptor superfamily [28]. Moreover, DA receptors can be divided into D_1-like receptors (D_1 and D_5) and D_2-like receptors (D_2, D_3 and D_5) based on its functions. Thus, D_1-like receptors can induce the production of cyclic adenosine monophosphate (cAMP) and activate cAMP-dependent protein kinase (PKA), and D_2-like receptors can reduce the accumulation of cAMP through interaction with G_i/G_0 proteins [30]. Additionally, D_2 has been reported to have two alternative splicing isoforms named short and long isoforms (D_2S and D_2L), which differ by only 29 amino acids, and which have been associated with different functions. Moreover, D_2S is expressed in the pituitary gland at higher levels than D_2L [31].

As mentioned above regarding SSTs, the functions triggered by DA depend on the specific subtypes and amount of DA receptors present in the tissue and the possible interactions between them and with other receptors such as SSTs, since heterodimerizations between these two types of receptors have been reported [32].

3.2.3 The pituitary as a metabolic sensor: Influence of metabolic cues in the control of pituitary function.

As mentioned earlier, not only central modulators but also a broad range of peripheral factors are involved in the precise control and regulation of pituitary hormone secretion under normal and pathological conditions. Among them, several reports have demonstrated that glucocorticoids are able to inhibit (in a short-time incubation) and stimulate (in a long-time

incubation) GH secretion [33, 34], and also to inhibit ACTH, PRL and TSH secretion *in vivo* and *in vitro* in humans and non-human primates [35, 36]. In the same line, thyroid hormones (T3 and T4) regulate TSH secretion through a direct negative feedback on pituitary gland [37], and also play a direct role in the regulation of somatotropes [38]. On the other hand, adipokines (leptin, resistin and adiponectin) comprise a family of cytokines mainly released from the adipose tissue. Adiponectin has been associated with the inhibition of GH and ACTH secretion and the stimulation of PRL release. Resistin can stimulate GH and ACTH secretion, and leptin can stimulate GH and FSH/LH release [39]. In the same line, free fatty acids (FFAs) have also been described as regulators of pituitary function. Thus, the elevation of plasma FFAs produces a strong inhibition of GH secretion in humans and non-human primates [40, 41]. Additionally, insulin and IGF1 can directly regulate somatotrope function under normal conditions [33], and IGF1 is also able to regulate PRL and TSH secretion [42, 43]. Because insulin and IGF1 are regulated by nutritional status, changes in circulating GH levels observed during starvation or obesity may in part be mediated by direct action of these hormone on somatotrope function [4]. In addition, other metabolic regulators involved in the control of pituitary gland are inhibins, activins, estrogens, testosterone, obestatin, follistatin, endothelin or opioids [4]. Therefore, based on all the information described above regarding the direct regulation of the function of different pituitary cells by multiple metabolic factors, it is reasonable to propose that pharmacological treatments currently used in the clinical practice to control metabolic alterations due to pathological conditions such as diabetes, obesity or hypercholesterolemia (e.g. metformin and statins), could also exert some actions at the pituitary gland and modulate its function under normal and pathological conditions.

3.3 Pituitary neuroendocrine tumors (PitNETs).

PitNETs represent approximately 15% of all brain tumors with a prevalence ranging from 1 in 865 persons to 1 in 2,688 persons [44, 45]. These tumors are usually accompanied by severe comorbidities related to mass effects and to an inadequate secretion of pituitary hormones, e.g. growth abnormalities, sexual dysfunctions, infertility, amenorrhea, galactorrhea, hypo- or hyperthyroidism, hypogonadism, hypopituitarism, etc. [5]. Although these tumors have been classically considered as a benign pathology (thus the term adenoma), because they very rarely metastatize, a recent consensus of the “International Pituitary Pathology Club” proposed a reclassification to accept the nomenclature “pituitary neuroendocrine tumors (PitNETs)” instead of “pituitary adenomas”. The main reason to propose this change is based

on the great number of behaviors of this pathology that are far from being “benign”, and in fact can cause severe comorbidities, despite the lack of metastasis. Additionally, the term “adenoma” is not considered appropriate to address invasive and aggressive pituitary tumors that cannot be surgically resected or controlled with therapy [46].

3.3.1 Pituitary tumorigenesis.

Several studies have reported that PitNETs arise from a monoclonal expansion of genetically altered cells, and that the external hypothalamic or peripheral factors could play a role potentiating their transformation [47-49]. Although the primary initiating cause of PitNETs remains unclear, several reports have demonstrated that there are numerous factors related with an increased proliferative potential of precursor cells for tumor formation and tumor growth, such as genetic or epigenetic events, paracrine growth factor disruption or even an altered intrapituitary microenvironment [49, 50]. Moreover, the use of transgenic mouse models has demonstrated that the overexpression or inactivation of cell cycle regulators is enough to trigger pituitary tumorigenesis **(Figure 3)** [51, 52].

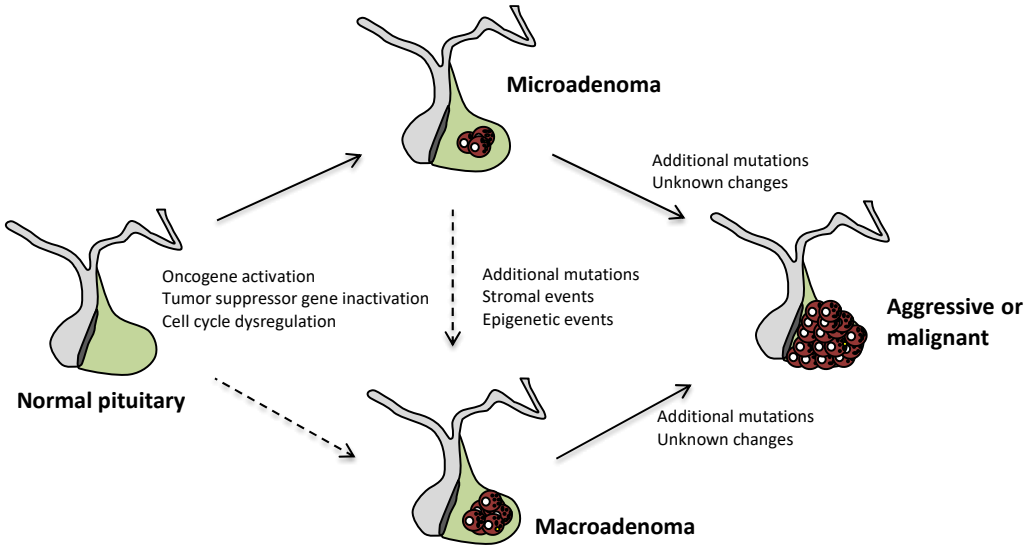

Figure 3: Cascade of pituitary tumorigenesis. Adapted from [49].

Although the classic oncogenes are rarely mutated in PitNETs [53], a growing set of pituitary-specific cellular disruptors has been described to be associated with pituitary tumorigenesis **(Figure 4)**. Importantly, the vast majority of PitNETs have a sporadic origin, but a small percentage (5%) are due to familial tumor syndromes such as Carney syndrome, familial isolated pituitary adenomas, multiple endocrine neoplasia type I or type 4, etc. [49, 54].

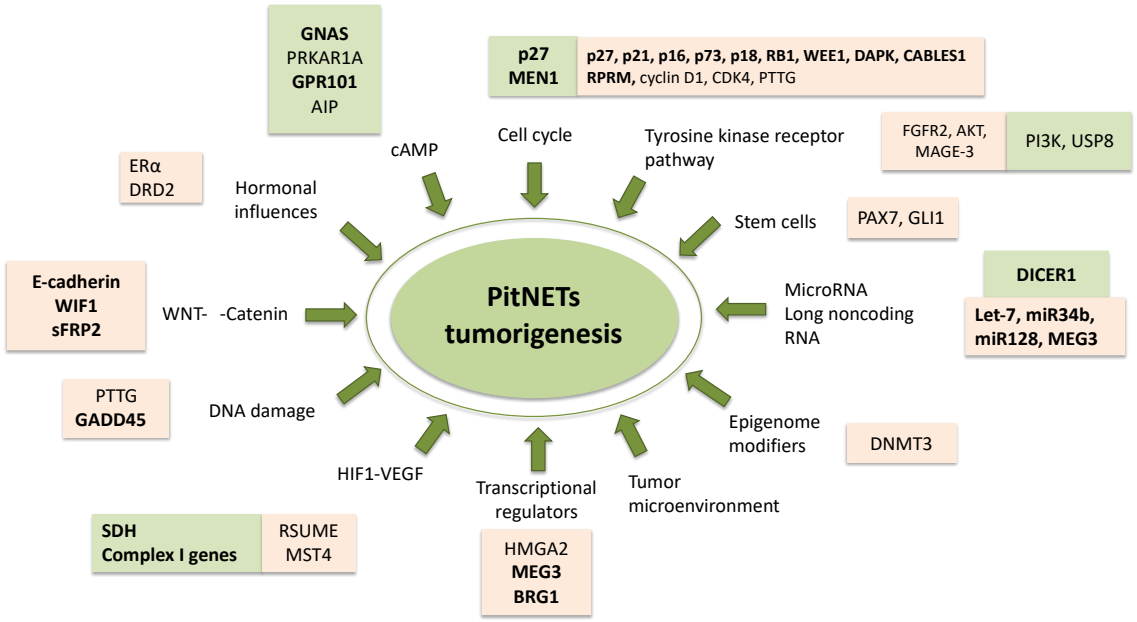

Figure 4: Main pituitary-specific cellular disruptors associated with molecular pathogenesis of PitNETs. Green boxes: pituitary tumorigenesis due to mutations (bold gene names represent tumor suppressor genes, gene names that are not bold represent oncogenes). Orange boxes: pituitary tumorigenesis due to altered gene expression. Adapted from [54].

In addition to all these disruptors, other epigenetic alterations can relevantly influence the development of tumors and cancer. Indeed, recent studies suggest that altered alternative splicing and, consequently, appearance of **abnormal or aberrant splicing variants**, is a common feature of most tumor pathologies, including PitNETs [23-25, 27, 55-58]. The cellular machinery that controls and carries out alternative splicing is the **spliceosome**, a ribonucleoprotein complex that recognizes specific sequences that determine the exact localization of the exon-intron junctions [59]. This complex machinery is comprised by proteins and ribozymes that act cooperatively in an extraordinarily dynamic fashion, and is organized into two systems, the major and the minor spliceosome. The major spliceosome is composed by a main core comprised by five small nuclear RNAs (snRNAs: U1, U2, U4, U5 y U6), which cooperate with more than 300 auxiliary proteins, the so-called splicing factors (i.e., SF2/ASF, RBM5 o FBP11), in the precise recognition of the target [60, 61] **(Figure 5)**. The minor spliceosome comprises U11, U12, U4atac, U5 and U6atac snRNAs. Thus, the splicing process is jointly executed by a discrete set of polypeptides associated with one or more snRNAs to form stable ribonucleoproteins (snRNPs), and multiple associated splicing factors comprising this intricate cellular machinery, which dynamically cooperate among them to finely regulate all the process [62]. Consequently, abnormal alteration of spliceosome function can compromise the natural splicing process of an ample range of genes, originating the appearance of

multiple, often aberrant splicing variants, which could be directly associated with the development/progression of tumor pathologies.

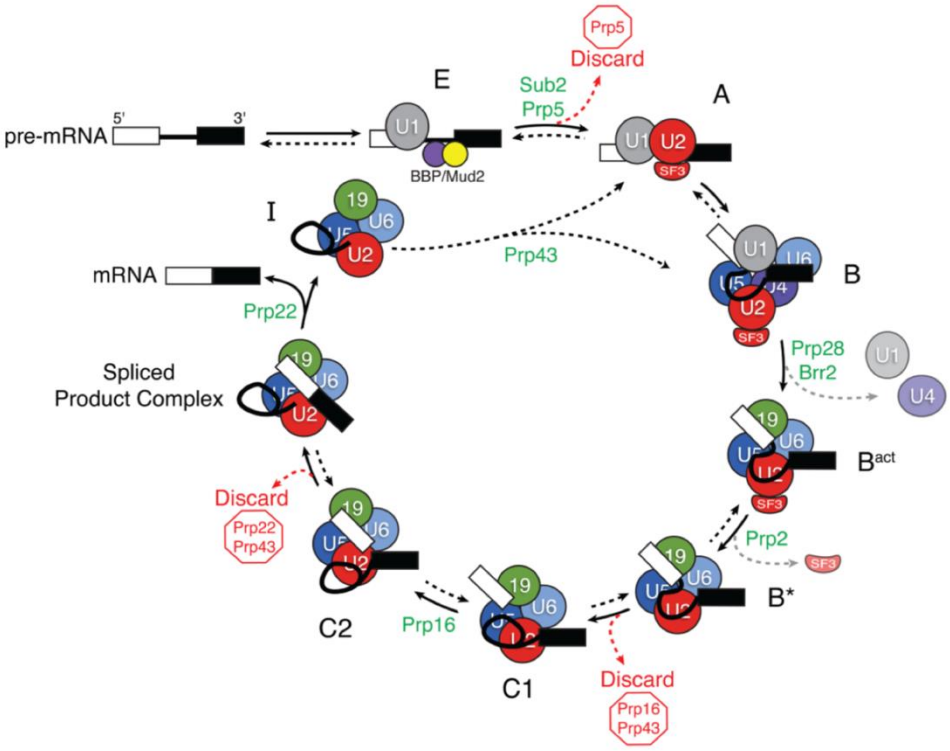

Figure 5: Model of spliceosome assembly and catalysis. Adapted from [59].

3.3.2 Classification of PitNETs.

PitNETs have been classically classified by combining histopathological features (e.g. hormone content) and ultrastructural characteristics (e.g. densely or sparsely granulated) of pituitary tumor cells [3, 63, 64]. However, the new classification proposed by the World Health Organization (WHO) has incorporated the role of transcription factors in tumor differentiation according to cellular lineage(s), regulation of specific pituitary hormone production, and possible tumorigenesis [44] **(Table 1)**. In addition to this canonical anatomo-pathological classification formulated by WHO, a prognostic clinicopathologic classification has recently been proposed based on large series of PitNETs [65]. This classification incorporates different parameters, including tumor size, tumor type, and tumor grade **(Table 2)** [65].

Table 1: Pathological classification of pituitary neuroendocrine tumors. Adapted from [44].

Tumor types	Morphological variants	Pituitary hormones by immunohistochemistry	Transcription factors and other co-factors
Somatotrope tumors	Densely granulated somatotrope tumor	GH, α-subunit	Pit-1
	Sparsely granulated somatotrope tumor	GH	Pit-1
	Mammosomatotrope tumor	GH + PRL (in same cells) ± α-subunit	Pit-1, ERα
	Mixed somatotrope-lactotrope tumor	GH + PRL (in different cells) ± α-subunit	Pit-1, ERα
Lactotrope tumors	Sparsely granulated lactotrope tumor	PRL	Pit-1, ERα
	Densely granulated lactotrope tumor	PRL	Pit-1, ERα
	Acidophil stem cell tumor	PRL, GH (focal and variable)	Pit-1, ERα
Thyrotrope tumors		β-TSH, α-subunit	Pit-1, GATA2
Corticotrope tumors	Densely granulated corticotrope tumor	ACTH	Tpit
	Sparsely granulated corticotrope tumor	ACTH	Tpit
	Crooke's cell tumor	ACTH	Tpit
Gonadotrope tumor		β-FSH, β-LH, α-subunit (various combinations)	SF-1, GATA2, ERα
Null cell tumor		None	None
Plurihormonal tumors	Pit-1-positive plurihormonal tumor (previously termed Silent subtype 3 tumor)	GH, PRL, β-TSH ± α-subunit	Pit-1
	Tumors with unusual immunohistochemical combinations	Various combinations	

The combined use of both WHO classifications and the a newly proposed comprehensive clinicopathological classification of PitNETs could provide an improved indicator for the clinical behavior of patients and to predict the probability of post- operative remission or tumor progression, which could help clinicians to choose the best post-operative therapy [65].

Table 2: Prognostic clinicopathologic classification of PitNETs. Adapted from [65]

Clinicopathologic classification of PitNETs
The classification is on the following 3 characteristics: 1. Tumor size into micro (<10 mm), macro (≥10 mm), and giant (>40 mm) by MRI 2. Tumor type into GH, PRL, ACTH, FSH/LH, and TSH by immunohistochemistry 3. Tumor grade based on the following criteria: - Invasion defined as histologic and/or radiological (MRI) signs of cavernous or sphenoid sinus invasion - Proliferation considered based on the presence of at least 2 of the following 3 criteria: Mitosis: n greater than 2 per 10 high-power field. Ki-67: greater than or equal to 3% p53: positive (>10 strongly positive nuclei per 10 high-power field The 5 grades are the following: **Grade 1a:** noninvasive tumor **Grade 1b:** noninvasive and proliferative tumor

Grade 2a: invasive tumor

Grade 2b: invasive and proliferative tumor

Grade 3: metastatic tumor (cerebrospinal or systemic metastases)

From a clinical point of view, approximately 65% of all PitNETs are functioning or hormone-secreting, whereas 35% are non-functioning tumors [66]. Non-functioning tumors or **non-functioning pituitary tumors (NFPTs)** comprise about 22-54% of all PitNETs and usually derive from cells of the gonadotrope lineage that synthesize, but not oversecrete, FSH and/or LH, although they can hypersecrete their common α-subunit [67]. In addition to those of a gonadotrope origin, there also NFPTs comprised by silent (containing but not oversecreting) ACTH, GH and rarely PRL cells [68]. NFPTs are often macroadenomas at diagnosis and are associated with secondary symptoms related to mass effects including headaches, hypopituitarism, visual defects and cranial nerve palsy [69]. On the other hand, a small proportion of gonadotrope-derived tumors are functional, and secrete FSH and/or LH. The clinical presentation of these **gonadotropinomas (FSH/LHomas)** is similar to NFPTs, but can also produce a mild or moderate elevation of prolactin levels due to pituitary stalk compression, and as rare manifestations may cause ovarian hyperstimulation, testicular enlargement or pituitary apoplexy [70]. **Prolactinomas (PRLomas)** comprise about 47-66% of all PitNETs and are associated with hyperprolactinemia, impaired fertility, decreased libido, amenorrhea, and galactorrhea. In addition, these tumors produce symptoms related to mass effects such as headache, visual disturbances and hypopituitarism [71]. **Somatotropinomas (GHomas)** arise from GH-secreting cells and represent 10-15% of PitNETs. Depending on the age of the patient, these tumors produce gigantism in children/adolescents, or acromegaly in adult patients, due to a GH hypersecretion, which generates an increase on IGF1 production by the liver. The main consequences of a long-term GH and IGF1 exposure include a number of severe morbidities such as acral changes, gigantism, prognathism, arthritis, osteopenia, vertebral fractures, organs growth, hypertension, heart disorders, diabetes, insulin resistance, hypogonadism, etc. [3, 72]. **Corticotropinomas (ACTHomas)** are composed by ACTH-secreting cells and represent 10-15% of all PitNETs. These tumors produce Cushing's disease (CD), a rare endocrine condition due to a chronic exposure to elevated glucocorticoid levels induced by ACTH hypersecretion. The clinical symptoms associated to these tumors can be also severe and include obesity, hypertension, diabetes, dyslipidemia, muscle weakness, and emotional disturbances, among others [3, 73]. **Thyrotropinomas (TSHomas)** account for less than 1% of all pituitary neoplasms. These tumors are diagnosed when TSH is unusually elevated or when is normal in a hyperthyroid patient with increased serum T4 levels. Most of them are large and

invasive at diagnosis, and present symptoms associated to mass effects (headache, visual defects and hypopituitarism) and thyroid dysfunctions [74]. Finally, **pituitary carcinomas** are very rare, as they account for just 0.2% of all PitNETs. These aggressive tumors are defined by the presence of craniospinal and/or systemic metastases. The early identification of these tumors is crucial since they are associated with higher morbidity and mortality [75].

3.3.3 Therapeutic options for PitNETs.

Surgical treatment is considered the first-line therapeutic option for patients harboring PitNETs, with the exception of PRLomas, where dopamine agonists are the treatment of choice [76]. Tumor removal is usually performed using transsphenoidal surgery (transnasal), whereas craniotomy is only recommended in patients with extrasellar tumors [77]. In this line, microscopy transsphenoidal approach has been classically used for PitNETs surgery. However, in the last decade, endoscopic transsphenoidal approach has been increasingly used to remove these lesions. In fact, endoscopic approach has been associated with higher gross tumor removal and lower incidence of septal perforation in patients with PitNETs compared to microscopic approach [78]. The surgical success is higher in patients with microadenomas (<10 mm), but in many cases there are an inadequate disease control after surgery and subsequent pharmacological therapy and/or radiotherapy is necessary.

3.3.3.1 Somatostatin analogues (SSAs).

Most PitNETs express SST_{1-5} at adequate levels to be responsive to SRIF or somatostatin analogues (SSAs), and consequently to reduce hormone secretion and, less frequently, cell proliferation [19, 79-81]. Although the potent and inhibitory effects of native somatostatin are widely known, its clinical usefulness is limited due to its short-half life (less than 3 min) [82]. Based on this information, synthetic SSAs were developed by different pharmaceutical companies **(Table 3)**. Thus, octreotide and lanreotide, analogues with preferential targeting to a single receptor, SST_2, and less affinity to SST_5 and the rest of SSTs, were the first generation of SSAs. These SSAs were first used for the treatment of GHomas and TSHomas to control hormone secretion, reduce tumor volume and ameliorate clinical symptoms [83-86]. In fact, octreotide and lanreotide have been largely used in the treatment of patients harboring PitNETs and other neuroendocrine tumors (NETs) [87, 88]. Nevertheless, a number of studies have reported that a proportion of patients are, or become, resistant or poorly responsive to these single-receptor-targeted analogues [19, 89]. Consequently, a second generation of SSAs with a multireceptor binding affinity was developed based on the notion that targeting several

SSTs simultaneously, like the natural ligand, SRIF, may be more effective in those unresponsive patients or those who escape to the therapy. The most studied compound in this group is pasireotide, which binds with high affinity to SST_5, SST_2, SST_3 and SST_1 , and is accordingly often referred to as a multi- of pan-SST agonist [90] **(Table 3)**. However, the biological action of SSAs does not solely depends on their biding affinity to the different SSTs. Indeed, pasireotide modulates intracellular trafficking of SSTs in a distinct manner than SRIF or octreotide, particularly producing a rapid recycling of SST_2 to the membrane after endocytosis. Instead, SST_3 is rapidly down-regulated after long-term exposure to SSA (octreotide or pasireotide), which suggests an early loss of response in PitNETs with a predominant SST_3 expression [91]. Interestingly, octreotide and pasireotide display both similar and distinct *in vitro* effects in primary cell cultures from PitNETs, and cell-type specific actions have been recently proposed for pasireotide, acting through SST_2 in patients responsive to first-generation SSAs and acting (also) through SST_5 in patients with unsatisfactory response to first-generation SSAs (low SST_2 expression levels) [92-94]. Moreover, although pasireotide has demonstrated high tolerability and efficacy in clinical trials, a hyperglycemia secondary to pasireotide treatment was found in most patients. Thus, patients under this treatment have to be monitored for glycemia control and treated with antidiabetic therapy [95].

Table 3: Binding affinities (IC_{50}) of somatostatin and somatostatin analogues. Adapted from [96, 97].

Compound	SST_1	SST_2	SST_3	SST_4	SST_5
SRIF-14	0.93	0.15	0.56	1.50	0.29
Octreotide	280	0.38	7.10	>1000	6.30
Lanreotide	2129	0.75	98	1826	12.7
Pasireotide	9.3	1.0	1.5	>1000	0.16

3.3.3.2 Dopamine agonists.

In addition to SSTs, PitNETs often express notable amounts of dopamine receptors (D_{1-5}), specially D_2 [80]. In line with this, DA agonists developed to selectively target for D_2 have been classically considered the first-line therapy for PRLomas [98]. Based on its chemical nature, DA agonists can be classified in two groups: the ergot derivatives (bromocriptine, pergolide and cabergoline) and the non-ergot derivatives (quinagolide). Bromocriptine was the first drug introduced 25 years ago into clinical practice to treat PRLomas. This treatment presents properties as D_2 agonist and D_1 antagonist and, importantly, demonstrated to control hyperprolactinemia in 70-90% of PRLomas and to reduce tumor size, although its use was

associated with several adverse effects. Even so, bromocriptine is prescribed as the best alternative to cabergoline in patients with hyperprolactinemia [76]. On the other hand, cabergoline is considered the therapy of choice to treat PRLomas, since this compound has higher affinity to bind to D_2 **(Table 4)**, fewer side effects, and a longer half-life than bromocriptine [30]. In fact, in a large study, cabergoline treatment controlled PRL levels in 86% of patients, reduced tumor mass in >80% of patients and even produce the total tumor disappearance in 26-36% of cases [76].

Table 4: Binding affinities (IC_{50}) of DA and its agonists. Adapted from [97].

Compound	D_2S	D_2L
Dopamine	350	320
Bromocriptine	4.5	3.9
Cabergoline	0.53	0.41

In this context, it is also worth to mention that D_2 is not only present in lactotropes, but is expressed in all pituitary cell types, and, accordingly, clinical and *in vitro* data suggest that cabergoline may also be effective as second-line of treatment in selected patients with persistent/recurrent Cushing's disease, acromegaly or NFPTs [99-101].

3.3.3.3 Chimeric compounds.

There is ample evidence that SSTs and DA receptors are highly expressed in most pituitary cell types. Interestingly, a number of reports have also demonstrated that certain subtypes of SSTs and DA receptors can form homodimers and heterodimers, which results in changes in the functional, pharmacological and signaling properties of the receptors involved [32, 102, 103]. This information, coupled to the fact that there are patients that are or become partially or totally unresponsive to classical SSAs, and that the combination of SSAs and DA agonists had been found to be more effective than the treatment with each individual compound in some cases, led to the concept that the development of chimeric compounds able to bind both SSTs and DA receptors could represent a promising therapeutic option to treat this type of patients. This led to the development of the so-called dopastatins. In particular, BIM-23A387, a chimeric SST_2-D_2 molecule **(Table 5)**, was the first drug developed to target both receptor families and demonstrated an enhanced potency to suppress GH secretion in GHomas compared with octreotide. Moreover, its higher efficacy was mainly attributed to its dopaminergic activity [104, 105]. Another compound of this class is BIM-23A760, the first termed as dopastatin, and was designed with higher binding affinity to bind

to SST_2, SST_5 and D_2 [106] **(Table 5)**. This compound can induce a greater suppression of GH secretion than octreotide in cell cultures from human partially responsive GHomas [106]. Moreover, in addition to its action on GHomas, BIM-23A760 demonstrated to control different pituitary hormonal secretions and to reduce cell proliferation and increase apoptosis in different PitNET subtypes, including ACTHomas, NFPTs or PRLomas [20, 107-109].

Table 5: Binding affinities (IC_{50}) of chimeric compounds. ND, not done. *IC_{50} for both D_2 isoforms. Adapted from [97, 104].

Compound	SST_1	SST_2	SST_3	SST_4	SST_5	D_2S	D_2L
BIM-23A387	293	0.1	77.4	ND	1000	22.1*	
BIM-23A760	622	0.03	160	>1000	42	15*	

However, treatment with BIM-23A760 also revealed the existence of two pituitary cell populations that oppositely responded to the drug (one showing an inhibitory response, and another with a stimulatory response) [109]. Moreover, a Phase IIb study using chronic administration of BIM-23A760 to acromegalic patients showed a profound dopaminergic effect that was caused by a metabolite accumulated in the circulation, which interfered with the activity of the parent compound [110]. For these reasons, BIM-23A760 was withdrawn from clinical development.

3.4 Non-human primates as suitable model to study pituitary physiology.

There is ample evidence that the quality and appropriateness of the experimental model employed to test a given scientific question represent the fundamental keys that determine the value and applicability of the results and information generated in research. To date, the vast majority of the information generated on the effects of different compounds on the function of normal pituitary gland and on their role in the proper control of normal pituitary physiology has been gathered in rodent models. However, this information is often not comparable, applicable or “translatable” to the human physiology, and in fact, in part due to this “species gap”, there are still a number of aspects about the regulation of human pituitary physiology that remain unclear. In this scenario, non-human primate models can provide a unique, close-to-human, normal pituitary model to investigate the direct actions of putative regulators of adenohypophyseal cell function. In line with this notion, our laboratory and others have used non-human primate models to achieve this valuable information, in that these samples may reproduce physiological conditions or indicate specific regulations of primate lineage that could not be determined with the use of other models [39, 111-116]. In particular, baboons (*Papio sp.*) and rhesus monkeys (*Macaca mulatta* and *Macaca fascicularis*)

are the most commonly used non-human primate models in biomedical research and results generated from these models are used to support and launch translational research to humans [117-119]. The main reason to use these models is based on the comparative genomic analyses, since their molecular phylogeny and the evolutionary process have revealed that the separation of this family from *Hominidae* family happened approximately 25 million years ago, which is relatively recent compared with the separation of rodent lineages that happened 65-85 million years ago [120, 121]. In addition, other evidence that support the use of these models is that *Macaca mulatta* and *Macaca fascicularis* show genetic identity of 93.54% and 92.83% with *Homo sapiens*, respectively [122, 123]. Therefore, all this information makes these species suitable models to study the effects of different compounds on normal pituitary cell function, which cannot be evaluated in healthy human subjects.

Aims of the study

4. Aims of the study

The general **aim of this study** was to extend our cellular, molecular and clinically-relevant knowledge on the (patho)physiological regulation of the pituitary gland, and of PitNETs, through the identification of novel factors and mechanisms involved in the functional response to different pharmacological therapies, including hormone release and the development and progression of pituitary tumor pathology.

To achieve this general aim, we proposed the following specific objectives:

Objective 1: To establish the precise effects of **biguanides** (metformin, buformin and phenformin) on pituitary cell function, by determining the direct *in vitro* impact of these compounds on different functional endpoints (hormone secretion, cell viability, apoptosis, cell signaling, etc.) in normal pituitary cells from two non-human primate species and in the most representative PitNET subtypes, and to determine the molecular mechanisms underlying those effects.

Objective 2: To determine the precise effects of **statins**, specially simvastatin, on pituitary cell function, by determining the direct *in vitro* impact of these compounds on different functional endpoints (cell viability, hormone secretion and cell signaling) in normal pituitary cells from a non-human primate species, in the most representative PitNET subtypes and in pituitary cell line models (AtT-20 and GH3), and to determine the molecular mechanisms underlying those effects.

Objective 3: To determine the therapeutic potential of targeting **SST_3** in PitNETs, by studying the *in vivo* and/or direct *in vitro* effects of different SST_3-specific agonists/antagonists on key functional parameters using primary cell cultures from non-functioning pituitary tumors, and that of a selected SST_3-specific agonist on tumor-growth in a preclinical mouse-model of PitNET.

Objective 4: To study the effects of a novel somatostatin/dopamine chimeric drug, **BIM-065**, on different types of PitNETs, and to compare its actions with those of currently used somatostatin analogues (octreotide and pasireotide), by evaluating different functional endpoints in cells derived from a well characterized set of PitNETs, and determining the molecular mechanisms underlying those effects.

Objective 5: To determine and analyze the expression levels of the **spliceosome** components and a selected set of relevant **splicing factors** in an ample and representative range of PitNETs and in normal human pituitary gland samples.

Results and General Discussion

5. Results and general discussion

5.1 Biguanides exert antitumoral actions in pituitary tumor cells through AMPK-dependent and independent mechanisms (Article I)

Metformin (MF), buformin (BF) and phenformin (PF) are antidiabetic drugs that belong to the family of biguanides. Currently, only MF is used in the clinical practice to treat type-2 diabetes mellitus (T2DM) [124]. In addition to its well-known anti-hyperglycemic effect [125], it has been suggested that biguanides, specially MF, may reduce the risk of cancer and tumorigenesis in different types of neoplasms such as brain, prostate, breast and NETs [126-131]. However, to date, the direct pharmacological effects of different biguanides on primary cell cultures from human PitNETs have been scarcely examined and are not fully elucidated. Therefore, we aimed to explore the direct effects of MF, BF and PF on key functional parameters (cell viability, apoptosis, hormone secretion/expression and intracellular signaling pathways) in primary cell cultures from different human PitNETs subtypes (13 ACTHomas, 13 GHomas, 13 NFPTs and 3 PRLomas), and two representative, widely use cell line models, the mouse corticotrope AtT-20 cells and the rat-derived somatotrope GH3 cells. In addition, we evaluated the effects of a combined treatment with MF and SSAs on cell viability and hormone secretion. Finally, clinical data of a second cohort of PitNETs were collected to explore the role of the pre-treatment with MF in patients with PitNETs.

In general, treatment with biguanides reduced cell viability in all PitNETs subtypes and decreased cell proliferation in the two pituitary cell lines analyzed. In particular, all biguanides reduced cell viability in ACTHomas, being PF the most effective compound, which is line with a recent report from our group in NETs [131]. Likewise, and consistent with a previous report [132], biguanides decreased cell proliferation in AtT-20 cells. In contrast, our results on GHomas are not totally in line with a previous report [133], in that we did not observe any alteration of cell viability in response to MF, whereas BF and PF clearly decreased cell viability. However, in GH3 cells, all biguanides reduced cell proliferation. Nevertheless, although we did not observe changes on cell viability in response to MF in GHomas, we detected an increase on apoptosis after 24h of incubation, which is consistent with results reported in GH3 cells [133]. These results might be viewed as somewhat contradictory; however, several studies have shown an imbalance between cell survival and apoptosis under pathological conditions [134-136]. In line with that found in GHomas, a similar pattern of response was found in PRLomas, where MF did not alter cell viability, while BF and PF decreased this parameter. These results could be due to the common developmental lineage of these cells [3], and differ

from the growth reduction observed in response to MF in the lactotrophic MMQ cell line [137]. In NFPT cultures, all biguanides reduced cell viability in a time-dependent manner.

SSAs constitute an important tool in the medical arsenal to treat some PitNET types, yet, in many cases, these drugs are or become ineffective [138, 139], especially when first-generation SSAs have been evaluated [140]. Thus, the search for new alternatives to control tumor growth and hormone secretion has been intensive over the last years. In this context, combination of SSAs with other pharmacological therapies (e.g. dopamine agonists or pegvisomant) are frequently used in PitNETs [141, 142]. For this reason, we tested the effects of combined administration of MF with SSAs (octreotide or pasireotide) in PitNETs primary cell cultures. However, this combined therapy did not enhance the inhibitory effect of MF or SSAs alone in ACTHomas and GHomas, which is reminiscent of the results reported using a combination of SRIF-14 and AICAR (AMP mimetic compound 5-aminoimidazole-4-carboxamide ribonucleoside) [143], and activator of AMPK, which is considered the central mediator of MF effects [144]. Conversely, the combination therapy displayed a stronger effect in reducing cell viability in NFPT cells. Although more experiments are necessary to confirm these results and to understand their mechanistic underpinnings, we speculate that this additive effect may offer a potential therapeutic avenue for patients with NFPTs and, hence, deserves further investigation.

In addition to cell viability/survival, we also explored the effect of biguanides on hormone secretion in PitNETs cells. Our results demonstrate that BF and PF, but not MF, clearly reduced GH and PRL secretion, but did not alter ACTH release, after 24h of incubation. These results compare nicely with those of a previous report from our group performed in two NET cell lines, BON-1 and QGP-1, in which MF did not modify hormone secretion in BON-1 or QGP-1 cell cultures, but PF decreased hormone secretion in BON-1 [131]. In contrast, all biguanides reduced GH secretion in the GH3 cell line after 24h of incubation, supporting the notion that the effects of biguanides are highly cell-type dependent. Additionally, we evaluated the combination of MF with SSAs on hormone secretion in GHomas. The results showed that the combination therapy did not enhance the inhibitory effect of SSAs as monotherapy.

Furthermore, we analyzed the direct effects of biguanides on mRNA levels of pathologically relevant genes in cell cultures from ACTHomas and GHomas. In contrast with our recent results in normal pituitary glands from non-human primate species (see section 3.2. below; [145]), MF and BF did not modify GH, POMC or SSTs mRNA levels. However, PF increased GH and SST_2 and SST_5 mRNA expression levels in a similar way to that seen in normal pituitary gland from *Papio anubis* [145]. These data indicate that the actions of

biguanides also include the regulation of the synthesis of key genes involved in the control of pituitary pathophysiology.

Additionally, our study also provides information about the signaling pathways underlying the effects of biguanides in PitNETs cells. In particular, our results revealed an inhibition on $[Ca^{2+}]_i$ levels in response to all biguanides in ACTHomas and NFPTs, but not in GHomas or PRLomas, suggesting that calcium kinetics in response to biguanides may be more related with their actions on cell viability than with the control of hormone secretion. Moreover, we found a regulation of phosphorylation levels of the key mediator of MF [144], AMPK in response to BF and PF, but not in response to MF, which is contrast with the results reported in GH3 and AtT-20 [132, 133, 146]. In line with this, we found an increase on phosphorylation levels of Akt and ERK1/2 in response to BF and PF, but not MF. These results suggest that some biguanides would act through AMPK-independent mechanisms in PitNETs cells, in a cell type-dependent manner.

Finally, to assess the clinical context of the potential interplay between PitNETs and biguanide treatment, we explore the putative association between treatment with MF and clinical outcomes of patients harboring different types of PitNETs. Specifically, we analyzed a cohort of 42 ACTHomas, 28 GHomas and 62 NFPTs available at the Reina Sofia University Hospital and compared relevant clinical characteristics between patients treated or not with metformin (see table 4 attached at the end of this manuscript). As shown, none of the clinical parameters evaluated (i.e. BMI, lesion type, tumor size, cephalea, visual alterations, extrasellar growth, presence of other treatments, persistence after surgery, etc.) revealed significant associations with MF use in this patient cohort.

In sum, the data presented herein demonstrate that biguanides exert distinct anti-proliferative and anti-secretory effects in specific PitNETs cell types, which would involve both AMPK-dependent ($[Ca^{2+}]_i$ kinetics and PI3K-Akt pathway) and -independent (ERK pathway) mechanisms. The combination therapy of MF with SSAs did not show relevant additive effects in most PitNETs, suggesting overlapping mechanisms of action. Of note, combined therapy might represent a potential therapeutic approach for NFPTs. Although MF use was not apparently related to any clinical variable in our cohort of patients, the limited number of patients treated with MF and the retrospective observational nature of the analysis may limit these results. Altogether, these data unveil a clear antitumoral effect of different biguanides on PitNETs cells and pave the way to further explore these compounds as a potential new option in the treatment of this pathology.

5.2 The pituitary gland is a novel major site of action of metformin in non-human primates: a potential path to expand and integrate its metabolic actions (Article II)

In addition to the well-known antihyperglycemic effect of MF, recent studies have reported that MF might exert additional beneficial actions both in the modulation of whole-body homeostasis and metabolism, by specifically targeting key endocrine/metabolic tissues, as well as by exerting other positive effects through alternative mechanisms of action (i.e. beneficial use in the treatment of cardiovascular diseases, aging, cancer, immune diseases, polycystic ovarian syndrome, etc.) [128, 130, 147-151]. To date, however, no studies have explored in detail how biguanides can modulate directly the function of the normal anterior pituitary gland in humans or in primate species, and what signaling pathways would be involved in those actions. In this sense, it is still under debate if AMPK is the central mediator of metformin in all of its metabolic actions, since AMPK-dependent and independent mechanisms have been proposed [144, 152, 153]. Therefore, we aimed to determine, for the first time, the direct effects of MF and PF on the secretion and gene expression of all anterior pituitary hormones in two non-human primate species [*Papio anubis* (baboons) and *Macaca fascicularis* (macaques)], and to understand the mechanisms behind the actions of these biguanides by evaluating the expression of key receptors and transcription factors, and by assessing different signaling pathways using standard pharmacological (inhibitory) approaches.

We first evaluated the direct effects of MF and PF on pituitary hormone release after short (4 h) and long (24 h) incubation periods. This revealed that both biguanides inhibited GH, ACTH and FSH release, but did not alter PRL, LH or TSH release, and also, that these inhibitory effects were more pronounced in a short-term incubation period. We then studied the direct interactions between MF and other primary regulators of pituitary function. Interestingly, MF was not able to inhibit the stimulatory actions of GHRH on GH release, of ghrelin on GH and ACTH release, and that of GnRH on FSH release, which suggest that MF and these regulators may operate through shared mechanisms of action, and also reveal that the inhibitory action of MF modulates pituitary cell function, but does not seem to overcome the stimulatory action of the primary hypothalamic regulators. On the other hand, our results on FSH are apparently in contrast with the only previous report indicating that GnRH-stimulated FSH/LH release was reduced by MF in rat pituitary cell cultures [154]. However, these discrepancies could be due to a number of different factors such as reproductive status of the donor, the time of incubation, cell preparation, but also to the fundamental differences in the physiology between rat and primate species.

In addition, we found that MF regulates not only hormone secretion, but also the synthesis of different hormones. Thus, MF treatment reduced GH and POMC mRNA expression levels after 24h of incubation, which would reinforce the reduction observed at the hormone release level. In contrast, PRL, FSH, LH and TSH expression levels were not altered by MF treatment, which also parallels the lack of effect of MF on the release of these hormones, with the exception of FSH. Thus, these data indicate that the direct pituitary actions of MF involve both hormone synthesis and release in somatotropes and corticotropes, whereas in gonadotropes it would only extend to vesicle release. Importantly, the inhibitory effects of MF and PF in both non-human primate species were not attributed to an alteration of cell viability or to the expression of the transcription factor Pit-1 since these parameters were not modified in response to the treatments.

To better understand these direct actions of MF, we also explored the intracellular signaling pathways that could underlie its inhibitory effects in primary pituitary cell cultures. Results from these studies, using a standard pharmacological (inhibitory) approach, indicated that the effects of MF on GH, ACTH and FSH release were mediated through mTOR, PI3K and intracellular Ca^{2+} mobilization, but did not require activation of adenylate cyclase (AC), phospholipase C (PLC) or extracellular Ca^{2+} mobilization. Additionally, the actions of MF on GH secretion also involved the MAPK pathway. Of note, although AMPK is often considered the central mediator of MF actions [144], we could not evaluate this pathway using the pharmacological approach due to lack of effective, and accepted, specific inhibitors of AMPK. Nevertheless, our data suggest that MF acts through AMPK-dependent mechanisms at pituitary levels since the signaling pathways that we found to be related to MF effects have been described to be upstream or downstream of AMPK signaling pathway [155-158]. However, we also observed that, in somatotrope cells, MF also acts through MAPK pathway, which is presumably not linked to AMPK, thus suggesting that MF also acts through AMPK-independent mechanisms in this cell type. The study of the signaling pathways shed some light on the possible reasons underlying the inability of MF to counteract the stimulatory actions of ghrelin, GHRH or GnRH. In this regard, it has been reported that ghrelin acts through PLC, protein kinase C (PKC), intracellular and extracellular Ca^{2+} mobilization, and MAPK at the pituitary level, while GHRH and GnRH act through AC/cAMP/protein kinase A (PKA), NOS/guanylate cyclase (GC) and intra-/extracellular Ca^{2+} dynamics [4]. Consequently, our results reveal that MF and those pituitary regulators act through both distinct and common (i.e. intracellular Ca^{2+} and MAPK pathway) signaling pathways, which may explain, at least in part, why MF did not influence the actions of these regulators at pituitary level.

Finally, our data also revealed that MF increases mRNA expression levels of key receptors of pituitary physiology (SST_2, SST_5, INSR and IGF1R), which probably are associated with inhibition of hormone secretion/expression in somatotropes, corticotropes and gonadotropes.

In summary, our results unveil that MF impacts directly on pituitary somatotrope, corticotrope and gonadotrope cells at the secretory, gene expression and signaling levels. These actions, if present *in vivo*, may have translational relevance. Indeed, the decrease on GH and ACTH secretion could be a beneficial metabolic action of MF to improve whole body homeostasis, inasmuch as several studies have reported that increased circulating levels of GH and glucocorticoids can lead to worsening of insulin resistance, glucose intolerance and diabetes mellitus [159-162]. Taken together, the results presented herein clearly suggest that the pituitary is a primary site for the pharmacological actions of MF, and that this gland would represent an additional, key target tissue and a true endocrine sensor contributing, in concert with other primary tissues (i.e. liver), to the well-known beneficial metabolic effects of biguanides in humans.

5.3 Simvastatin exerts antitumoral actions in pituitary tumor cells (Article III)

Statins are well-stablished drugs commonly used to treat hyperlipidemia, and cardiovascular and coronary heart diseases. In addition to the cholesterol-lowering effects, statins have been related with a broad range of pleiotropic effects [163, 164]. Among them, numerous reports have described anticancer effects of statins in an ample selection of tumor types, including neuroendocrine tumor cells [131, 165-168]. However, to the best of our knowledge, the precise role of statins in PitNETs cells has not been explored hitherto. Therefore, we aimed to ascertain the direct effects of statins, specially simvastatin, on important functional parameters (cell viability/proliferation, hormone secretion and intracellular signaling pathways) in primary cell cultures from different human PitNETs subtypes, including ACTHomas, GHomas and NFPTs, as well as in two representative pituitary model cell lines from rodents, the AtT-20 corticotropes and the GH3 somatotropes. Moreover, we also analyzed the ability of simvastatin to influence relevant functional parameters (hormone secretion, cell viability, gene expression and intracellular signaling pathways) in primary cell cultures from normal pituitary of baboons (*Papio anubis*).

The first approach of our study was to evaluate the effect of different statins on cell proliferation, a parameter tightly linked to tumor growth. In general, all statins were able to significantly decrease cell proliferation in AtT-20 cell line, with simvastatin showing slightly stronger effects. These results compare favorably with the statin-induced reduction of cell

proliferation reported in murine pheochromocytoma cell lines [167] and in human pancreatic neuroendocrine tumor cell lines [131]. In the same line, our results showed a clear decrease on cell viability at different times of incubation in different PitNETs subtypes. Interestingly, the effect of simvastatin was stronger in ACTHomas and NFPTs compared with GHomas, which are the most resistant tumor types to the currently available therapeutic options [93, 169]. Most importantly, treatment with simvastatin (24h) did not alter cell viability in normal pituitary cells. Furthermore, we also explored the effect of simvastatin on hormone secretion in different PitNETs subtypes and in normal pituitary cells. Thus, simvastatin treatment (24h) reduced ACTH, GH, and chromogranin-A (CgA) secretion in ACTHomas, GHomas, and NFPTs, respectively, and ACTH, GH, PRL, FSH and LH secretion in normal pituitary cells. These results are also of potential clinical relevance, in that hypersecretion of pituitary hormones causes serious comorbidities in patients with PitNETs [3]. Hence, a reduction of hormone secretion from tumor cells in response to simvastatin would be clearly beneficial for patients harboring these pathologies.

Regarding the signaling pathways underlying these effects, our results showed that inhibitory actions of simvastatin in ACTHomas and NFPTs, but not in GHomas or normal pituitary cells, might be slightly mediated through the mobilization of $[Ca^{2+}]_i$ levels. Moreover, we also found a reduction on the phosphorylation levels of ERK1/2 in response to simvastatin after a short-term (4h) and after a long-term (24h) incubation in AtT-20 and GH3 cells, respectively. In the same line, we use a standard pharmacological (inhibitory) approach to evaluate the signaling pathways involved in simvastatin effect in normal pituitary cells. Specifically, we found that the reduction of ACTH, GH, PRL, FSH and LH was mediated through mTOR and PI3K pathways, but not through intracellular or extracellular Ca^{2+} influx. Moreover, inhibitory actions on ACTH, GH and PRL secretion also require MAPK, which is in accordance with the results observed in AtT-20 cells. Remarkably, simvastatin also evoked a downregulation in the expression of key receptors associated to primary stimulation of ACTH, GH, PRL, FSH and LH secretion (i.e. GHRHR, GNRHR and KISS1R), which might also be serving to enhance the inhibitory effects of simvastatin on hormone release from corticotrpes, somatotropes, lactotropes and gonadotropes observed herein.

On the other hand, taking in account that there are numerous clinical trials, ongoing or recently finished [170], using statins as antitumor agents, and that we have reported antitumor actions of metformin in NETs and PitNETs [131, 171], we next studied the combination therapy of simvastatin and metformin in PitNETs cell lines. In general, combined treatment did not modify the effects of simvastatin tested alone (with the exception of an additive effect at 24h in GH3 cells that disappeared at 48-/72-h). Likewise, SSAs are also

frequently used alone or in combination with other pharmacological options (e.g. dopamine agonists, pegvisomant) to control PitNETs symptoms [141, 142]. In our study, the combination of simvastatin with SSAs did not produce any additive effect in AtT-20 or GH3 cells. These results might suggest that simvastatin, MF, and SSAs are sharing mechanisms of action to exert their inhibitory effects in pituitary tumor cells. Indeed, several reports have associated the activation of AMP-activated protein kinase (AMPK; considered the central mediator of MF actions) by statins in numerous cancer types [165, 172, 173], and also, statins have been linked with the dysregulation of several pathways like Ras-MAPK, PI3K-Akt, and AMPK/Akt/mTOR, all of them also involved in the effects of SSAs and MF [174, 175].

Finally, we also explored the putative association between treatment with statins and clinical outcomes of patients harboring different PitNETs subtypes. Specifically, we analyzed an independent cohort of 42 ACTHomas, 28 GHomas and 62 NFPTs available at the Reina Sofia University Hospital. This revealed that patients treated with statins show a trend to have less extrasellar growth compared with patients not treated with statins ($p=0.07$). In contrast, none of the other clinical parameters evaluated (i.e. BMI, lesion type, tumor size, cephalea, visual alterations, presence of other treatments, persistence after surgery, etc.) revealed significant associations with the use of statins in this patient cohort.

In conclusion, our study provides primary evidence that statins, specially simvastatin, exert important antiproliferative and antisecretory effects in different PitNETs subtypes, without altering the viability of normal pituitary cells. Furthermore, we present mechanistic insights into the plausible signaling pathways underlying the inhibitory actions of simvastatin on cell survival and hormone release, which may partially overlap with those employed by MF or SSAs, as their combination with simvastatin did not elicit relevant additional antitumor effects. Therefore, our data unveil clear antitumoral direct effects of simvastatin on PitNET cells, thereby opening the possibility to explore these compounds as a novel tool to enrich the limited pharmacological arsenal available to treat patients harboring PitNETs.

5.4 A novel SST_3 agonist shows potential antitumor effects in experimental models of Nonfunctioning Pituitary Tumors (Article IV)

Currently, the available pharmacological treatment approaches for PitNETs (mainly SSAs and dopamine agonists) have shown very limited efficacy in NFPTs [77], and are therefore, sparingly applied. In the case of SSAs, it has been proposed that this lack of efficacy may be due to the low SST_2-SST_5 expression levels as compared to those of SST_3 [176, 177], given the

preferred targeting of first generation SSAs (octreotide, lanreotide) for the former receptor subtypes. Importantly, SST_3 has been associated with apoptotic/antiproliferative actions in several studies using cell lines and, in fact, apoptotic actions of SRIF/SSAs have been historically related to SST_3 [19, 178]. In this context, pasireotide, which also shows high affinity for SST_3, has been proposed as a potential therapeutic option in the treatment of NFPTs [177]; but, recent results from our group demonstrates that pasireotide does not exert a clear inhibitory effect on primary cultures from NFPTs [93]. Based on this information, we hypothesized that a compound that selectively or preferentially binds to SST_3 might be an effective therapeutic option to treat NFPTs. However, it has been very challenging to find peptides that selectively activate SST_3, and thus, the functional role and pathophysiological relevance of this receptors in NFPTs is still poorly understood. Accordingly, the aims of this study were to identify specific SST_3 agonists through the screening of a peptide library; and to determine the *in vitro* and *in vivo* therapeutic potential of SST_3 agonists. To this end, we first studied the direct effect of the identified SST_3-specific agonists and antagonists on key functional parameters for NFPTs, including cell viability, caspase activity, chromogranin-A (CgA) secretion, signaling pathways, and mRNA levels, and also examined the functional consequences of *SSTR3* silencing on cell viability. Furthermore, we evaluated the effect of a selected SST_3-specific agonist on tumor-growth in a preclinical mouse-model of PAs.

We first screened a library of synthetic-peptides produced at IPSEN selecting for compounds that bind to SST_3 and applying a multidimensional Spotfire analysis to identify compounds with the highest selectivity for SST_3. Thus, SST_3 binding affinities and functional cAMP assays revealed that BIM-355 and BIM-071 were the most potent SST_3-agonists, and BIM-839 was the most potent SST_3-antagonist.

Next, we corroborated that SST_3 was overexpressed in our cohort of patients, and demonstrated that SST_3 (mRNA and protein levels) could discriminate between NFPTs vs functioning pituitary tumors (FPTs), but not between normal pituitaries (NPs) and FPTs.

In addition, we explored the functional role of SST_3 using the new peptidic SST_3 agonists and antagonists in comparison to the previously published non-peptidic L-796,778 SST_3 agonist. Our results revealed a general ability of SST_3 agonists to reduce cell viability in NFPT cells, being BIM-355 the most potent compound, causing a 23.2% of reduction, which, to our knowledge, is the most prominent reduction reached on cell viability through the activation of SST_3 [178]. We also found an increase on caspase activity in response to SST_3 agonists, which is in line with previous results in HEK-293 cell line using L-796,778 [178]. These effects on caspase activity, as well as those on cell viability, were completely blocked by the antagonist BIM-839, thus confirming the specificity of the actions observed. Moreover, supporting these

results, we demonstrated, for the first time, that *in vivo* administration of an SST_3-specific agonist significantly decreased tumor growth in a preclinical mouse model of PitNET [179].

Our data also revealed a decreased of CgA secretion in response to SST_3 agonists in NFPT cells. CgA has been considered as a useful marker of pharmacological effectiveness and has been recently proposed as a biomarker of tumorigenesis and invasiveness [180, 181]. Interestingly, our *in vitro* approach unveiled that a subset of NFPTs did not respond to SST_3 agonists in terms of inhibition of cell viability, which is not unexpected since several reports have shown a substantial proportion of unresponsive PitNETs upon treatments with different compounds [93, 138]. A comparison of the expression profile of SSTs revealed that high SST_3 expression levels are a requisite to elicit a significant response after SST_3 agonists administration, which is also in line with the finding that ROC-curve analysis demonstrates that SST_3 is able to discriminate between responsive and unresponsive NFPTs. Moreover, treatment with SST_3 agonists increased SST_3 expression levels in responsive, but not in unresponsive NFPTs, thereby suggesting the existence of a positive homologous SST_3 (receptor)-*SSTR3* (gene) regulatory circuit in these tumors.

An additional set of studies was aimed at identifying the signaling pathways underlying the ability of SST_3 agonists to generate functional responses. Specifically, NFPT cells showed a scarce and infrequent response to agonists in terms of $[Ca^{2+}]_i$ kinetics. In contrast, in responsive NFPTs, BIM-355 produced a striking reduction on the phosphorylation levels of relevant protein kinases associated to three important signaling pathways: MAPK, PI3K-AKT/mTOR and JAK/STAT. Indeed, we found a decrease on ERK1/2 and JNK phosphorylation among other proteins of the MAPK pathway in responsive, but not in unresponsive NFPTs. These results are in line with previous results on HEK-293 cells treated with L-796,778 [178]. Similarly, we also observed a reduction on phosphorylation levels of Akt, GSK-3a/b, Src, PRAS40, mTOR and p70-S6 kinase, all of them associated to the PI3K-AKT/mTOR pathway, which controls cell cycle progression, protein synthesis and cell proliferation [182]. Additionally, we observed a reduction on the phosphorylation levels of some key components of the JAK/STAT signaling pathway, such as STAT3, STAT5a/b and STAT6. Although GPCRs have not been traditionally related with this signaling pathway, there are several reports describing an activation of STAT3 by GPCRs associated with cancer progression [183, 184]. Conversely, of note, the phosphorylation status of most of the proteins described above were not altered or were increased in unresponsive NFPTs. These results compare favorably with results reporting an increase on phosphorylation of Akt levels in a proportion of NFPTs resistant to rapamycin [185] and with results in PRLomas showing that a reduced D2S/D2L ratio and an increase on MAPK and PI3K/AKT/mTOR pathways might contribute to tumorigenesis and DA resistance

[186]. In addition, the comparison of basal phosphorylation levels of responsive and unresponsive NFPTs revealed a clear pattern of inhibition in the basal levels of the MAPK, PI3K-AKT/mTOR and JAK/STAT proteins in unresponsive tumors, which could explain the resistance of this population of NFPTs to respond to the SST_3 agonist (and possibly to other SSAs).

Consistent with the existence of two populations of NFPTs, the silencing of *SSTR3* gene expression by two specific siRNAs (corroborated at mRNA and protein levels) increased cell viability by 38% of NFPTs. A potential explanation for this result would be that SST_3 bears a constitutive inhibitory activity, inasmuch as previous results have demonstrated that various SSTs, including SST_3, display a relevant degree of ligand-independent constitutive activity in different pituitary cell systems [187, 188]. Another possibility would be that these cells release SRIF or cortistatin. In this regard, our results revealed negligible levels of SRIF and much higher levels of cortistatin, which might suggest the existence of an ultra-short autocrine feedback mechanism whereby activation of SST_3 by endogenous cortistatin would mediate inhibition of cell viability, secretion, etc. Inversely, a reduction in SST_3 protein levels (by silencing) could lead to the loss of this inhibitory loop and could, ultimately, induce an increase on cell viability. Importantly, the fact that BIM-355 did not alter cell viability in s13501-transfected cells (with reduced SST_3 content), together with the fact that SST_3-antagonist BIM-839 completely blocked the effects of SST_3-agonists, provide compelling evidence that the effects observed in response to agonists are completely dependent on and only specific for SST_3.

Altogether, this study represents the first identification and characterization of potent and selective SST_3 peptidic agonists, which enabled to gain novel experimental results supporting that these agonists exert clear antitumor actions on NFPT primary cell cultures. Therefore, the present study provides new, compelling evidence demonstrating that SST_3 has a functional role in the pathophysiology of NFPTs, and invites to suggest that pharmacological treatments specifically targeting this receptor could become a promising option to treat patients with NFPTs.

5.5 A new generation somatostatin-dopamine analogue exerts potent antitumoral actions on pituitary neuroendocrine tumor cells (Article V)

The development of chimeric compounds able to bind simultaneously to SSTs and Ds provided a novel, promising therapeutic approach to treat different PitNETs subtypes. However, results published regarding BIM-23A760, a chimeric compound able to bind to SST_2, SST_5 and D_2, revealed the existence of two PitNET cell populations that oppositely respond to the treatment, referred to as an inhibitory- and a stimulatory-population [109], suggesting that

this compound might not be a successful therapy in some patients with PitNETs. For this and other pharmacologically-related reasons, a new generation of chimeric agonist for $SST_2/SST_5/D_2$ receptors (named BIM-065), with higher potency, efficacy and safety has been recently designed and developed by IPSEN that may be used for clinical purposes in the future. Therefore, the main aim of this study was to evaluate, for the first time, the direct effects of this new compound on relevant functional parameters such as cell viability, apoptosis, hormonal secretion and expression and $[Ca^{2+}]_i$ kinetics, in primary cell cultures from different PitNETs, including ACTHomas, GHomas, NFPTs and PRLomas.

ACTHomas represent a suitable model to test the effect of BIM-065 since its preferred target receptors are highly expressed in these tumors ($D_2T>D_2L>SST_5>>SST_2$). We measured the expression of these receptors and also that of the two truncated SST_5 variants, SST_5TMD4 and SST_5TMD5, since the presence of these isoforms have been associated to a poor response to SSAs [22, 23]. However, the expression levels of these truncated variants were negligible in this cohort of PitNET samples. To our knowledge, this is the first time that the effect of a chimeric compound is evaluated in detail in ACTHomas. Our results revealed that BIM-065 induced a comparable reduction of cell viability than octreotide at 10^{-7} M, but the reduction achieved at 10^{-9} M was higher in response to BIM-065. We also found that BIM-065 increased apoptosis and inhibited ACTH secretion after 24h of incubation, without altering mRNA levels of key genes (POMC, SST_2, SST_5, D_2, PTTG, CDKN1B or CDK2). Likewise, we observed a reduction on $[Ca^{2+}]_i$ levels in all ACTHomas tested, which is in line with results observed in response to BIM-23A760 [109], although the percentage of responsive cells was higher with BIM-065. Additionally, to further test the capacity of BIM-065 to induce functional responses, we measured several components of two important signaling pathways (MAPK and PI3K-Akt) in corticotrope AtT-20 cells. These pathways are tightly linked to cell growth, proliferation and survival in tumor pathologies, including PitNETs [175, 189]. Interestingly, short-term incubation with BIM-065 (10 min) clearly increased p-Akt, which was followed by a clear reduction of the anti-apoptotic protein Bcl-2. In contrast, long-term incubation with BIM-065 (24h) numerically up-regulated p-ERK1/2 levels, although this latter effect did not reach statistical significance. A similar stimulatory response in ERK1/2 levels has been previously reported in PitNET cells after treatment with BIM-23A760 and the dopaminergic agonist BIM-53097, which was mainly associated to an activation of the dopaminergic signaling through D_2 [107]. Therefore, these results might suggest that BIM-065 modulate ERK1/2 and Akt through the preferential activation of the D_2/dopaminergic-signaling. Moreover, the decrease in Bcl-2 levels support the increase of apoptosis observed in response to BIM-065 in PitNETs.

Similar to ACTHomas, GHomas express high levels of SSTs and Ds ($D_2T>D_2L>SST_5>>SST_2$). Moreover, BIM-065 produced a higher reduction of cell viability and a comparable increase of apoptosis compared with our previous results using BIM-23A760 [109]. However, BIM-065 produced a more striking reduction of GH secretion compared to BIM-23A760 or other chimeric compounds [105, 109, 190]. These results are in agreement with a recent report showing that BIM-065 can reduce GH and IGF1 levels in healthy male volunteers [191]. Comparison of GH reduction between BIM-065 and SSAs did not show any relevant difference. Nevertheless, further experiments with a higher number and variety of tumor samples will be necessary to unequivocally stablish whether BIM-065 is more potent and efficacious than SSAs in GHomas. Additionally, the new dopastatin evoked a clear reduction on $[Ca^{2+}]_i$ levels, which also agrees with the inhibitory response observed in response to BIM-23A760 [109].

In the course of the study, we had the opportunity to test the new chimeric compound on two PRLomas. Interestingly, we observed clear differences in the response to BIM-065 and cabergoline in terms of cell viability between the two PRLomas analyzed, which could be due to their differential receptor expression pattern. Thus, the tumor that was highly responsive expressed higher levels of SST_5 compared to SST_2 and higher levels of both isoforms of D_2. Furthermore, these data are in line with previous results reporting that dopaminergic contribution is more important for the response to chimeric compounds, such as BIM-23A760, than the expression of SST_2 [192]. Consistent with previous results observed with chimeric compounds [109, 190] and with BIM-065 in healthy male volunteers [191], BIM-065 produced a decrease on PRL release. Obviously, additional experiments are necessary to confirm and extend these results, and to explore the signaling pathways involved in the effects exerted by BIM-065 in PRLomas.

NFPTs expressed high levels of SST_2, SST_5 and D_2 ($D_2T>D_2L>>SST_2>SST_5$) and negligible levels of truncated SST_5TMD4 and SST_5TMD5. However, and in contrast with previous results with other dopastatins [108, 109], BIM-065 did not produce any detectable alteration on cell viability. On the contrary, BIM-065 induced a clear increase of apoptosis and a decrease in CgA in all cases tested. Although the vast majority of observations suggest that the cell proliferation and programmed cell death are effectively coupled [193, 194], there are several studies showing an imbalance between cell proliferation/survival index and apoptosis in pathological conditions [134, 135]. Nevertheless, further experiments are required to understand why cell proliferation and apoptosis are apparently uncoupled in response to BIM-065 in NFPTs cell cultures.

In sum, our study provides compelling evidence demonstrating that BIM-065 can directly alter cell function and behavior in different PitNETs subtypes. Thus, BIM-065 acted mostly by

reducing cell viability and hormone secretion and increasing apoptosis. Importantly, in contrast to previous data with other dopastatins, treatment with BIM-065 did not evoke any stimulatory action in the tumor cells analyzed, while the proportion of responsive tumors/cells was higher with this compound than with BIM-23A760, suggesting that this novel dopastatin is a more efficacious and specific chimeric compound, an may become an attractive and valuable tool in the future treatment of PitNETs. Thence, further studies should be developed to confirm and expand the original results provided herein, and to better understand the intracellular mechanisms underlying the effects of BIM-065 since this new generation chimeric compounds may hopefully help to enhance the currently scarce pharmacological arsenal for the treatment of patients harboring PitNETs.

5.6 Splicing machinery is dysregulated in pituitary neuroendocrine tumors and associated with aggressiveness features (Article VI)

the primary initiating cause of PitNETs development and possible the existence of general and distinctive signatures and molecular elements in this heterogeneous pathology is still under debate. In this scenario, an emerging body of evidence indicates that altered alternative splicing and its consequent outcome, i.e. the appearance of abnormal patterns of splicing and even that of aberrant splicing variants, represents a common feature across most tumor pathologies, including PitNETs [23, 25, 55-58]. In addition, the spliceosome system is becoming an attractive therapeutic target for tumor pathologies [195]. This is the case for pladienolide-B, a natural compound that directly targets and binds a key player in the spliceosome, SF3B1, and thereby inhibits spliceosome functions, which in turns appear to mediate the antitumor properties of this promising drug [195, 196]. However, the expression pattern and putative role of the core splicing machinery components in the development and progression of PitNETs, as well as the potential therapeutic effects of pladienolide-B in PitNET cells, has not be reported. For these reasons, we aimed to determine and analyze the expression levels of the spliceosome core components and a selected set of relevant splicing factors (SFs) in the main PitNETs subtypes (GHomas, NFPTs, ACTHomas and PRLomas) as compared to normal human pituitary gland samples. Additionally, we evaluated the potential antitumor actions of pladienolide-B in PitNET cells by evaluating key functional parameters (i.e. cell proliferation/viability and hormone secretion) in human primary PitNETs cell cultures and pituitary cell lines (AtT-20 and GH3).

One of the main observations of this study is the fact that the spliceosome machinery is dysregulated in a tumor subtype-dependent manner, where NFPTs, GHomas, ACTHomas and

PRLomas exhibit a differentially altered pattern of expression. Specifically, NFPTs showed a profound downregulation of three major spliceosome components, two minor spliceosome components and 15 SFs compared to normal pituitaries. Bioinformatics analyses [i.e. Variable Importance in Projection (VIP) score of Partial Least Squares-Discriminant (PLS-DA)] revealed that SRSF9, SND1, U2AF1 and CELF4 were the components with higher capacity to discriminate between NFPTs and NPs. Moreover, ROC curve analyses of these four components corroborated their capacity to discriminate between NFPTs and NPs showing an AUC of 0.94, 0.94, 0.93 and 0.89, respectively ($p<0.001$). Interestingly, a clear alteration of these spliceosome components found in our cohort of NFPTs has also been observed in other tumor pathologies. Specifically, SRSF9 and SND1 have been found to be overexpressed and associated with an increase in cell proliferation, invasion and poor prognosis in several tumor pathologies [197-200]. Moreover, CELF proteins have been reported to regulate the splicing of neurofibromatosis type 1 (NF1) protein generating a protein with the exon 23a excluded, which has 10 times greater ability to regulate Ras signaling, a main component of MAPK signaling pathway [201]. In addition, U2AF1 is an important component of the major spliceosome that has been found frequently mutated and associated to the generation of particular splicing patterns in several pathologies [202, 203]. In this sense, our results might suggest that not only the mutation pattern but also the expression pattern of U2AF1 could be involved in the malignant behavior of tumor pathologies including NFPTs.

In GHomas, analysis of a cohort of acromegalic patients from Spain (cohort-1) showed a significant overexpression of 6 major spliceosome components, a downregulation of one component of the minor spliceosome and 17 significantly altered SFs (16 upregulated and one downregulated). In this case, we found that the expression levels of three SFs (RAVER1, RBM3 and SRSF6) with the highest score in the VIP analysis were able to discriminate between GHomas and NPs in two perfect clusters. Moreover, ROC curve analyses of these three SFs showed an AUC of 0.99, 1, and 0.98, respectively. Additionally, we confirmed the overexpression of RAVER1 and RBM3, but not SRSF6, in another independent cohort of GHomas (tumors from Brazil; cohort-2). Interestingly, the dysregulation of RAVER1 and RBM3 has been recently associated with the development of non-alcoholic fatty liver disease and/or with the development of type-2 diabetes in patients with cardiovascular disease [204, 205]. Moreover, RBM3 and SRSF6 have been associated with aggressive features and with poor prognosis in several tumor pathologies [206-208], and SRSF6 has been postulated as a possible therapeutic target to reduce tumorigenesis in colorectal cancer [206-208].

In ACTHomas, our data revealed a significantly dysregulation of 11 splicing machinery components, two components of major spliceosome and 9 altered SFs. Further analysis

revealed that the pattern of two SFs with the highest score in the VIP analysis (MAGOH and KHDRSB1) was able to discriminate between ACTHomas and NPs in two perfect clusters, and ROC curves analyses of those factors corroborated this capacity with an AUC of 1 and 0.97, respectively. These results are in accordance with the overexpression of KHDRSB1 found in gastric, epithelial ovarian cancer or sacral chordomas, wherein its presence was associated with poor prognosis and aggressive characteristics [209, 210]. Likewise, MAGOH has been shown to be differentially expressed in breast cancer, where it served, together with other RNA processing factors, to develop a robust stratification of breast cancer subtypes [211]. However, the presence and potential role of KHDRSB1 and MAGOH in PitNETs or normal pituitary has not been reported hitherto.

In addition, PRLomas exhibited an overexpression of three major spliceosome components, a downregulation of one minor spliceosome component, and an alteration of 14 SFs (12 upregulated and 2 downregulated) in comparison with NPs. However, the combination of the components with higher score in VIP analysis (RNU11, ESRP2, RNU6ATAC, SRSF1 and ESRP1) was not sufficient to clearly distinguish between both populations. This might probably be associated to the low number of PRLomas analyzed in this study due to the difficulty to have access to this type of samples since dopamine agonist's treatment regimens are highly successful in patients with PRLomas.

Interestingly, we also pinpointed a common downregulation of three minor spliceosome components (RNU11, RNU4ATAC and RNU6ATAC) and one SF (SRSF1) in PitNETs, irrespective of their origin, which might be patho-physiologically relevant. The fact that these spliceosome components are similarly dysregulated in all PitNETs, despite the high heterogeneity of these tumors, invite to speculate about the existence of common driver alterations in pituitary tumorigenesis, which would pave the way toward the identification of common therapeutic targets based on the dysregulations of these key elements. However, further studies should be conducted to test this hypothesis.

Finally, our study also demonstrates that the pharmacological disruption of the splicing process using pladienolide-B could have potential antitumor actions in patients with different types of PitNETs. Thus, this compound significantly inhibited cell viability/proliferation in all PitNETs and cell lines analyzed, and also decreased GH secretion after 24h of incubation in GHomas. Our results are in line with the antitumor actions of this compound (i.e. reduction on cell viability and colony formation) previously observed in *HeLa* cells [196]. However, it should be mentioned that NFPTs were less sensitive to the effect of pladienolide-B compared to GHomas, result that is in line with previous observations in response to other pharmacological treatments in NFPTs [93, 169, 171].

Altogether, our data provide compelling evidence to propose that the splicing machinery is severely and distinctly dysregulated in the main subtypes of PitNETs compared to NPs, and identified unique fingerprints of spliceosome components in each PitNETs subtype that can accurately discriminate between normal and tumor pituitary tissues. Furthermore, we also found several components, including SFs (SRSF1) and specially three minor spliceosome components (RNU11, RNU4ATAC and RNU6ATAC), commonly dysregulated in all PitNET subtypes, which could represent novel, common therapeutic targets in these pathologies. These discoveries open a new window to investigate the plausible contribution of splicing dysregulation and its subsequent outcomes to pituitary tumorigenesis, and to assess the potential value of specific splicing machinery components as novel diagnostic/prognostic tools in these pathologies. Furthermore, our study unveils splicing, particularly SF3b1, as a novel actionable therapeutic point that can be targeted by Pladienolide-B to combat PitNETs.

Conclusions

6. General conclusions

1) Biguanides (metformin, buformin and phenformin) reduce cell proliferation and hormone secretion in specific PitNETs cell types and these effects seem to involve AMPK-dependent (Ca^{2+}_i signaling and PI3K-Akt pathway) and -independent (MAPK pathway) mechanisms. The combined administration of MF and SSAs did not exert additive effects in functioning PitNETs, suggesting an overlap of their signaling mechanisms. In contrast, this combined therapy exerted stronger effects in reducing cell viability in NFPT cells. Altogether, these results pave the way to further explore biguanides as a potential treatment in PitNETs.

2) Metformin exerts direct regulatory effects on pituitary cells by acting at both hormone secretion and gene expression levels in somatotrope, corticotrope and gonadotrope cells, in a cell-type specific manner, and through mechanisms involving common and distinct signaling pathways. These results suggest that the pituitary gland is a primary site for the pharmacological actions of metformin, thereby reinforcing the view that this gland represents a true endocrine-metabolic sensor, and would serve as an additional, key target tissue contributing, in concert with other primary tissues (i.e. liver), to the well-known beneficial metabolic effects of metformin in humans.

3) Simvastatin exerts antiproliferative and antisecretory effects in different PitNETs subtypes mainly through the modulation of MAPK pathway, without altering cell viability of normal pituitary cells. Simvastatin also reduces hormone secretion in normal cells through MAPK, mTOR and PI3K pathway. Combination of simvastatin with MF or SSAs did not result in additive effects in tumor pituitary cell lines. Our findings unveil clear antitumoral effects of simvastatin and open the way to also consider these drugs as a potential valuable tool to treat patients with PitNETs.

4) Selective SST_3 agonists decrease cell viability and chromogranin-A secretion and increase apoptosis in primary cell cultures from NFPTs, by causing a reduction in the phosphorylation of key targets associated to MAPK, PI3K-Akt/mTOR and JAK/STAT pathways. Moreover, the SST_3-specific agonist BIM-355 reduced tumor growth in a preclinical mouse model of PitNET. Taken together, these results demonstrate that SST_3 plays a relevant functional role in the pathophysiology of NFPTs and suggest that pharmacological treatments specifically targeting this receptor could become a promising option to treat patients harboring NFPTs.

5) The dopastatin BIM-065, a new chimeric compound targeting $SST_2/SST_5/D_2$, can directly reduce cell viability and hormone secretion and increase apoptosis in different subtypes of PitNETs by modulating MAPK and PI3K-Akt pathways, likely through the

preferential activation of D_2/dopaminergic signaling. In contrast with previous chimeric compounds, this dopastatin did not produce any stimulatory action in the tumor cells analyzed, and the proportion of responsive tumors/cells observed was higher, which suggest that this novel compound may become a valuable tool in the future treatment of PitNETs.

6) The splicing machinery is clearly dysregulated in all the main PitNETs subtypes as compared to NPs. Furthermore, this dysregulation provides specific fingerprints of spliceosome components and SFs capable to accurately discriminate between each PitNET subtype and NP tissue. Interestingly, this approach identified several components, including minor spliceosome components and relevant splicing factors, that are commonly dysregulated in all PitNETs analyzed, and could unveil novel and common tumor drivers and potential therapeutic targets in these tumors. Actually, our results also indicate that pharmacological blockade of the splicing process with pladienolide-B exerts clear antiproliferative and antisecretory actions in PitNETs cells.

In conclusion, when viewed together, the results from this thesis indicate that novel therapeutic avenues to combat PitNETs can derive from the repurposing of commonly used metabolic drugs, such as metformin or simvastatin, which could be a promising tool given their antitumoral properties described herein. Moreover, new treatment approaches for PitNETs can arise from the development of novel compounds innovatively targeting key regulatory receptors for somatostatin and dopamine, such as SST_3-specific agonists or novel chimeric SST_2/SST_5/D_2 compounds, where the precise knowledge of the quantitative expression profile of those receptors might be a key molecular feature to precisely and predictively select the most adequate therapeutic option to treat PitNETs in the future. Finally, our study provides primary evidence that the splicing machinery is profoundly dysregulated in PitNETs, and some its components could be involved in pituitary tumorigenesis and may offer new tools to identify original diagnostic and prognostic biomarkers and to explore unprecedented therapeutic avenues in PitNETs. Hence, we consider that this thesis clearly illustrates the notion that the application of innovational molecular, multidisciplinary and collaborative approaches to investigate the biology of the pituitary gland and the pathophysiology of PitNETs enable the discovery of valuable information on the functioning of this “master gland” and provides new instrumental insights to improve our capacity to detect and combat PitNETs.

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7. References

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List of Articles

8. Articles

This Thesis is based on the research articles and review listed below.

Article I. Biguanides exert antitumoral actions in pituitary tumor cells through AMPK-dependent and -independent mechanisms.

Article II. The pituitary gland is a novel major site of action of metformin in non-human primates: a potential path to expand and integrate its metabolic actions.

Article III. The pituitary gland is a major site of actions of statins: Potential antitumor effects of simvastatin in pituitary neuroendocrine tumor cells.

Article IV. A novel SST_3 agonist shows potential antitumor effects in experimental models of non-functioning pituitary tumors.

Article V. A new generation somatostatin-dopamine analogue exerts potent antitumoral actions on pituitary neuroendocrine tumor cells.

Article VI. Splicing machinery is dysregulated in pituitary neuroendocrine tumors and associated with aggressiveness features.

Article VII. Multiple signaling pathways convey central and peripheral signals to regulate pituitary function: Lessons from human and non-human primate models (Review).

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Biguanides exert antitumoral actions in pituitary tumor cells through AMPK-dependent and -independent mechanisms.

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Direct effects of biguanides on pituitary tumors.

Biguanides exert antitumoral actions in pituitary tumor cells through AMPK-dependent and -independent mechanisms.

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Context: Pituitary neuroendocrine tumors (PitNETs) comprise a commonly underestimated pathology in terms of incidence and associated morbimortality. Currently, an appreciable subset of patients is resistant or poorly responsive to the main current medical treatments [i.e. somatostatin-analogues (SSAs)/dopamine-agonists]. Thus, development/optimization of novel/available medical therapies is necessary. Biguanides (metformin/buformin/phenformin) are antidiabetic drugs that exert antitumoral actions in several tumor types, but their pharmacological effects on PitNETs are poorly known.
Objective: We aimed to explore the direct effects of biguanides on key functions (cell-viability/hormone-release/apoptosis/signaling-pathways) in primary cell-cultures from human PitNETs and cell-lines. Additionally, we evaluated the combination of metformin with SSAs on cell-viability and hormone secretion.
Design: A total of 13 corticotropinomas, 13 somatotropinomas, 13 non-functioning PitNETs, 3 prolactinomas and two tumoral pituitary cell-lines (AtT-20 and GH3) were used to evaluate the direct effects of biguanides on cell-viability, hormone-release, apoptosis and signaling-pathways.
Results: Biguanides reduced cell-viability in all PitNETs and cell-lines (being phenformin the most effective biguanide), and increased apoptosis in somatotropinomas. Moreover, buformin and phenformin, but not metformin, reduced hormone secretion in a cell-type specific manner. Combination metformin-SSAs therapy did not enhance SSAs monotherapy effectiveness. Effects of biguanides on PitNETs could involve the modulation of AMPK-dependent ($[Ca^{2+}]_i$, PI3K/Akt) and independent (MAPK) mechanisms.
Conclusion: Altogether, our data unveil clear antitumoral effects of biguanides on PitNET cells, opening new avenues to explore their potential as drugs to treat these pathologies.

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Biguanides reduced aggressiveness features (proliferation, apoptosis, etc.) in pituitary neuroendocrine tumors, opening new avenues to explore their potential as drugs to treat these pathologies.

INTRODUCTION

Pituitary neuroendocrine tumors (PitNETs) are mostly benign neuroendocrine tumors that comprise a commonly underestimated pathology in terms of incidence and associated morbimortality. Specifically, PitNETs constitute approximately 15% of all intracranial neoplasms and appear in approximately 16.9% of the population (1-3). The genesis of PitNETs resides in an excessive and uncontrolled cell proliferation produced by the expansion of precursor cells. PitNETs are often accompanied by serious comorbidities related to mass effects and inappropriate secretion of pituitary hormones (1, 4-6). Transsphenoidal surgery is the first-line therapy in these patients, but approximately a 30% show tumor regrowth after surgery (7). The pharmacological arsenal currently available to treat PitNETs is mainly limited to synthetic somatostatin analogues (SSAs) and dopamine agonists (8), which exert their effects through the binding to their corresponding G-protein coupled receptor families, both encoded by 5 genes (*SSTR1-5* and *DRD1-5*, respectively) (9, 10). These drugs have a demonstrated efficacy in decreasing hormone hypersecretion and inducing tumor shrinkage/stabilization in functioning PitNETs (8, 11). However, some patients are (o become) unresponsive to these drugs (9, 12). For these reasons, the search for new therapies to control tumor growth and/or hormone secretion is crucial.

Metformin (MF), buformin (BF) and phenformin (PF) are antidiabetic drugs belonging to the biguanide family. Currently, only MF is used to treat type-2 diabetes mellitus (T2DM) (13). In addition to its well-known anti-hyperglycemic effect (14), it has been suggested that MF may reduce the risk of cancer and tumorigenesis in different types of neoplasms such as brain, prostate, breast and neuroendocrine tumors (15-20). Based on this, clinical trials using MF in non-diabetic cancer patients have been performed. Unfortunately, some results are contradictory, especially in terms of reduction of Ki-67 expression, but other findings seem to be promising, including the reduction of the prostate specific antigen in prostate cancer patients (15). The precise molecular mechanisms underlying the antitumoral effects of MF are still controversial. Specifically, MF activates the AMP-activated protein kinase (AMPK), which has been associated to the modulation of cell proliferation, hormone secretion and apoptosis in endocrine-related cancers and in PitNET cells (21, 22). Additionally, MF has been also suggested to exert some of its actions through AMPK-independent mechanisms (23).

Recent results from our group have revealed that biguanides reduce viability and secretory activity in two neuroendocrine tumor model cell lines (20). Moreover, we have also reported recently that both MF and PF exert notable direct effects in the modulation of hormonal secretion in normal pituitary cells from two primate species (24). In line with our observations, some recent reports have provided evidence that MF has a direct effect altering certain functional parameters in somatotrope GH3 and corticotrope AtT-20 pituitary cell lines (25-27). However, to date, the pharmacological effects of different biguanides on human primary PitNET cell cultures are not fully elucidated. Therefore, we aimed to explore the direct effects of MF, BF and PF on key functional parameters (cell viability, apoptosis, hormonal secretion/expression and intracellular signaling pathways) in primary cell cultures from different human PitNETs subtypes, including adrenocorticotropin and growth hormone secreting adenomas (ACTHomas, GHomas, respectively), non-functioning pituitary adenomas (NFPAs) and prolactinomas (PRLomas).

MATERIALS AND METHODS

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Drugs and reagents

All reagents and drugs used in this study were purchased from Sigma-Aldrich unless otherwise specified. Buformin was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Octreotide was obtained from GP-Pharm (Barcelona, Spain) and pasireotide was generously provided by Novartis (Barcelona, Spain). Metformin was used at 10 mM, buformin and phenformin at 5 mM, and SSAs at 100 nM. All doses were selected based on previous studies (20, 24, 28, 29) or based on *in vitro* dose-response experiments (see Figure 1).

Patients, samples and primary cell cultures

This study was carried out within a project approved by our Hospital Research Ethics Committee and was conducted in accordance with ethical standards of the Helsinki Declaration of the World Medical Association. Written informed consent was obtained from each patient. Human pituitary samples were collected during transsphenoidal surgery from 42 patients (13 corticotropinomas, 13 somatotropinomas, 13 NFPAs and 3 prolactinomas). General characteristics of the patients are summarized in Table 1. In all cases, specimens were placed in sterile cold medium (S-MEM, Gibco, Madrid, Spain; supplemented with 0.1% BSA, 0.01% L-glutamine, 1% antibiotic-antimycotic solution, and 2,5% HEPES) and dispersed into single cells following the methods previously described (29-32). The type of tumor was confirmed by 2 separate methods: examination by anatomo-pathologists and molecular screening by quantitative real-time PCR (qPCR), as previously described (29-32).

Cell lines and culturing

The two pituitary cell line models most widely used in cell biology research were used in the present study: the mouse corticotrope pituitary derived cell line AtT-20/D16v-F2 (ATCC® CRL-1795™) and the rat somatotrope pituitary derived cell line GH3 (ATCC® CCL-82.1™). Both were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) complemented with 10% FBS, 100 U/ml penicillin/streptomycin, 0.024 M of 2-(4(2-hydroxyethyl)-1-piperazine)-ethane sulfonic acid (HEPES), and maintained at 37ºC and 5% CO2, under sterile conditions. Additionally, both cell lines were checked for mycoplasma contamination by PCR (33).

Analysis of cell viability

As previously reported (30, 32), Alamar-blue reagent (Invitrogen, Madrid, Spain) was used to assess the effect of different biguanides alone, or the combination of MF with SSAs (octreotide or pasireotide) every 24h until 72h on cell viability. 10,000 cells/well (human pituitary cultures) or 6,000 cells/well (AtT-20 and GH3 cell cultures) were plated in a 96-well plate. Treatments were daily refreshed after each measurement and cell viability was evaluated using FlexStation III system (Molecular Devices, Sunnyvale, CA, USA).

Measurement of hormone release

To analyze the effect of different biguanides alone, or the combination of MF with SSAs (octreotide or pasireotide) on pituitary hormone release from different primary PitNET cells and/or cell lines, 150,000-200,000 cells/well were plated onto 24-well plates in serum-containing media. Media were collected after 24h of incubation and hormone secretion was measured using human [reference numbers: ACTH: EIA-3647; GH: EIA-3552; PRL: EIA-1291 (DRG, Mountainside, NJ, USA)], and rat [reference number: GH: EZRMGH-45K (Merck Millipore, Darmstadt, Germany)] commercial ELISAs, following the manufacturer´s instructions.

Analysis of apoptotic rate in somatotrope cells

Caspase-Glo 3/7 assay (Promega, Madrid, Spain) was used to analyze the effect of MF on apoptotic rate by measuring caspase 3/7 activity according to manufacturer's instructions.

Thus, 25,000 cells/well were plated in 96-well white microplate and maintained for 24h at 37ºC and 5% CO_2. Then, cells were treated with MF and vehicle and incubated for another 24h. After this period, 100 µl of Caspase-Glo 3/7 reagent was added to each well and luminescence was measured at room temperature using FlexStation III system for 3h.

RNA isolation, reverse transcription and qPCR of human and primate transcripts

Details of RNA extraction, quantification, reverse-transcription (RT) and qPCR using a specific set of primers included in this study have been previously reported elsewhere by our group (31, 32). New primer sequences were used in the present study to amplify rHprt1 (sense, AGCTTGCTGGTGAAAAGGAC and antisense, TCCACTTTCGCTGATGACAC; accession number, NC_005120.4; product size, 153pb), rPpia (sense, CGTCTGCTTCGAGCTGTTT and antisense, GGAACCCTTATAGCCAAATCCT; accession number, NC_005113.4; product size, 97 pb), $rSst_1$ (sense, TGCCCTTTCTGGTCACTTCC and antisense, AGCGGTCCACACTAAGCACA; accession number, NC_005105.4; product size, 135 pb), $rSst_2$ (sense, CCCATCCTGTACGCCTTCTT and antisense, GTCTCATTCAGCCGGGATTT ; accession number, NC_005109.4; product size, 134 pb), $rSst_5$ (sense, TCATTGTGGTCAAGGTGAAGG and antisense, AAGAAATAGAGGCCGGCAGA; accession number, NC_005109.4; product size, 199 pb), rAmpk (sense, CTGTAAACACGGGAGGGTTG and antisense, ACGTTCTCTGGCTTCAGGTC; accession number, NC_000070.6; product size, 120 pb), mAmpk (sense, TCGGCTGGTTGTAGTGAATG and antisense, TCTCCTTCTGTTTGGCACCT; accession number, NC_000071.6; product size, 106 pb) and hAMPK (sense, AGATTGTATGCAGGCCCAGA and antisense, TGGTCATCATCAAATGGAAGG; accession number, NC_000005.10; product size, 92 pb). To control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, the expression level (copy-number) of each transcript was adjusted using a normalization factor (NF) calculated from beta actin (ACTB), hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels in PitNETs and calculated from Actb, Hprt and Ppia (peptidylprolyl isomerase A) expression levels in cell lines.

Measurement of free cytosolic calcium ($[Ca^{2+}]_i$) kinetics

Kinetics of free cytosolic calcium ($[Ca^{2+}]_i$) were measured in response to different biguanides alone in human pituitary primary cell cultures, as previously described (30-32). Thus, 50,000 cells/coverslip were plated and changes in $[Ca^{2+}]_i$ in single cells were measured using fura-2AM (Molecular Probes, Eugene, OR, USA).

Measurement of signaling pathways by western blotting

In short, 500,000 cells/well were cultured in 12-well plates and incubated for 8 min with different biguanides alone and vehicle-treated controls. Proteins were extracted, separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Darmstadt, Germany), as previously reported (31). Then, blocked membranes were incubated with the primary antibodies [anti-phosphoAMPKα (SC-33524) and anti-ERK1/2 (SC-154) from Santa Cruz (CA, USA) and anti-AMPKα (2532S), anti-phosphoERK1/2 (4370S), anti-Akt (9272S) and anti-phosphoAkt (4060S) from Cell Signaling (Danvers, MA, USA)] and appropriate secondary antibody (anti-rabbit antibody from Cell Signaling, Danvers, MA, USA). Proteins were developed using an enhanced chemiluminescence detection system (GE Healthcare, Madrid, Spain) with dyed molecular weight markers. A densitometric analysis of the bands was carried out with ImageJ software. Relative phosphorylation was estimated from normalization of p-AMPK, p-ERK1/2 or p-Akt against the total AMPK, ERK1/2 or Akt, respectively.

Statistical analysis

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Statistical differences were evaluated by paired parametric t-test or two-way ANOVA followed by Tukey's test for multiple comparison (according to normality evaluated by Kolmogorov-Smirnov test). All data are expressed as mean ± SEM. As previously reported (30), to normalize values within each treatment and minimize intragroup variations in the different *in vitro* experiments (i.e. different age of the tissue donor or metabolic environment), the values obtained were compared with vehicle-treated controls (set at 100%). All experiments were performed in a minimum of three independent primary pituitary cultures from different patients (3-4 replicates/treatment per experiment), unless otherwise indicated. P-values ≤0.05 were considered statistically significant. A trend for significance was indicated when p-values ranged between >0.05 and <0.1. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software; La Jolla, CA, USA).

RESULTS

Biguanides reduce cell viability in PitNET cells

In general, administration of biguanides produced a reduction of cell viability in all types of PitNET cell cultures and a decrease in cell proliferation in the pituitary cell lines tested (Fig-2). In particular, MF (10 mM) significantly reduced cell viability after 72h of incubation in ACTHomas cells (Fig-2A), and BF and PF (5 mM) also decreased cell viability in ACTHomas after 24, 48 and/or 72h of incubation, being PF the most effective compound (Fig-2A). Moreover, MF (the only type of biguanides currently used in the clinical practice) was also evaluated in combination with SSAs (octreotide or pasireotide) in primary ACTHoma cultures (Fig-2B). Thus, MF alone significantly reduced cell viability by 49.7% after 72h of incubation. In contrast, although treatment with octreotide and pasireotide alone apparently reduced cell viability at 72h (35.1% and 18.4% reduction, respectively), this reduction did not reach statistical significance in any case (Fig-2B). Co-incubation of MF with both SSAs did not significant alter the inhibitory actions of MF (Fig-2B). In the same line, MF significantly reduced cell proliferation after 72h of incubation in AtT-20 cells (Fig-2A). BF and PF (5 mM) also decreased cell proliferation in AtT-20 cells after 24, 48 and 72h of incubation (Fig-2A), being PF the most effective compound (Fig-2A). In this point, it should be clarified that Fig. 2B only shows the ACTHoma samples that were treated with metformin, SSAs and the combination of them (n=3 experiments), whereas Fig. 2A shows the results of all the ACTHoma samples treated with metformin (n=10 cell cultures), including the tumors that appeared in Fig. 2B. Therefore, although the final results and conclusion are similar in both graphics (i.e. metformin treatment significantly decreased cell viability in primary cell cultures from ACTH-producing tumors), direct comparisons between figures 2A and 2B should be done with caution, as the number of tumors included in each figure differs considerably.

In primary GH-secreting PitNET cell cultures, administration of MF did not alter cell viability at any of the incubation times tested; whereas, in contrast, BF, but specially PF, clearly decreased cell viability after 24-72h of incubation (Fig-2C). In the GH3 cell line, all biguanides significantly decreased cell proliferation (Fig-2C). Co-administration of MF with SSAs was also evaluated in primary GH-secreting PitNET cells (Fig-2C). As previously observed, MF did not alter cell viability in GHoma cell cultures; however, octreotide and pasireotide alone decreased cell viability by 35.8% and 33.2% at 72h of incubation, respectively. The combination therapy of MF with octreotide or pasireotide did not alter the inhibitory effect of both SSAs (Fig-2D).

In primary NFPA cell cultures, treatment with MF and BF significantly decreased cell viability after 48-72h of incubation and PF decreased this parameter after 24-72h of incubation in a time dependent-manner (Fig-2E). Interestingly, although cell viability was not reduced in response to octreotide and pasireotide in NFPA cell cultures, combination therapy

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of MF with both SSAs seemed to produce a higher decrease on cell viability as compared to the different treatments alone (Fig-2F); however, this reduction did not reach statistical significance probably due to the fact that we could only test this combination therapy in two primary NFPA cell cultures. Finally, PF, but not MF and BF, significantly decreased cell viability in primary PRLoma cell cultures (Fig-2G).

Metformin increases apoptotic rate in GHoma cells.
Due to the limited number of cells obtained after dispersions, the effect of MF on apoptosis was only evaluated in primary GH-secreting adenomas. Specifically, MF (10 mM) significantly increased caspase 3/7 activity, a robust indicator of apoptosis, as compared to vehicle-treated controls after 24h of incubation (Fig-2H).

Effect of biguanides on hormone secretion in PitNET cells.
MF, BF and PF did not alter ACTH secretion after 24h of incubation in ACTHoma cell cultures (Fig-3A). In GHoma cell cultures, BF and PF, but not MF, reduced GH secretion (Fig-3B). In line with the cell viability results previously observed, the three biguanides significantly reduced GH secretion in the GH3 cell line after 24h of incubation (Fig-3E). Additionally, we had the opportunity to measure the effect of MF alone or in combination with SSAs (octreotide and pasireotide) in cell cultures from GHomas (Fig-3C). The results showed that treatment with SSAs alone tended to decrease GH release and, that the combination therapy of SSAs with MF did not modify the inhibitory action of both SSAs when tested alone (Fig-3C). Finally, BF and PF, but not MF, treatment tended to decrease PRL secretion in cell cultures from PRLomas as compared to vehicle treated-controls after 24h of incubation (Fig-3D).

Effect of biguanides on mRNA expression of relevant genes in PitNETs.
We evaluated the direct effect of the treatment with different biguanides alone on mRNA levels of pathologically relevant genes in cell cultures of corticotropinomas (primary ACTHoma cells and AtT-20 cells) and somatotropinomas (primary GHoma cells and GH3 cells). MF and BF did not modify the mRNA expression levels of POMC (ACTH-precursor) in ACTHoma or AtT-20 cell cultures (Fig-4A); in contrast, PF significantly reduced Pomc expression levels in AtT-20, but not ACTHomas, cell cultures (Fig-4A). Interestingly, PF, but not MF or BF treatment tended to increase the expression levels of somatostatin receptors (SST_1, SST_2 and SST_5) in ACTHoma cell cultures (Fig-4B; left-panel). Similar results were observed in AtT-20 cell cultures wherein PF significantly increased Sst_2 expression levels (both isoforms identified in rodents) but not Sst_5 [Fig-4B; right-panel; Sst_1 was not expressed in AtT-20 cell line (data not shown)].

Administration of PF, but not MF or BF, increased GH mRNA levels after 24h of incubation in GHoma and GH3 cell cultures (Fig-4C). Interestingly, treatment with PF, but not MF and BF, increased SST_2 and SST_5, but not SST_1 expression levels in GHoma cell cultures (Fig-4D; left-panel). In GH3, Sst_2 expression seemed to be up-regulated in response to the treatment with all biguanides, although this increase was only statistically significant in response to MF. Moreover, PF increased Sst_5 and reduced Sst_1 expression levels (Fig-4D; right-panel).

To further explore the mechanisms involved in biguanide actions, we measured the effects of MF, BF and PF on the expression of AMPK, since this enzyme has been typically considered the central mediator of MF effects (34). Results revealed that administration of MF and BF did not modify AMPK expression in ACTHoma or GHoma cell cultures (i.e. primary ACTHoma and GHoma cell cultures and AtT-20 cells, but with the exception of GH3 wherein MF increase Ampk expression levels). By contrast, PF treatment significantly increased AMPK expression levels in all the cellular model of ACTHoma or GHoma tested (Fig-4E and -4F, respectively).

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Effect of biguanides on intracellular signaling pathways in PitNET cells.

To test the ability of biguanides to modulate intracellular signaling pathways in PitNETs, we first evaluated the dynamics of free cytosolic calcium concentration ($[Ca^{2+}]_i$) in single cells derived from ACTHomas, GHomas, NFPAs and PRLomas in response to the treatment with MF, BF and PF (Table 2). Specifically, all biguanides elicited a calcium response in more than 50% of ACTHomas analysed, evoking a similar reduction in $[Ca^{2+}]_i$ that ranged between 23.34 and 27.11%. However, differences were found regarding the percentage of responsive cells. Thus, BF was the most effective compound since 46.80% of ACTH-secreting cells responded compared to 30.25% or 9.8% of responsive cells to PF and MF, respectively. In contrast, treatment with biguanides did not elicit any $[Ca^{2+}]_i$ response in the GHomas analyzed. In NFPAs, MF reduced $[Ca^{2+}]_i$ by 30.62% in 50% of the samples, BF decreased it by 20.6% in 66.7% of NFPAs and PF was the most effective compound reducing $[Ca^{2+}]_i$ by 44.7% in 32.4% of responsive cells of the 66.7% NFPAs analyzed. Finally, in PRLomas, BF and PF did not alter $[Ca^{2+}]_i$ dynamics and MF reduced by 18.4% in one of the two tested PRLomas.

Furthermore, we could analyze the combination therapy with octreotide in 2 ACTHomas and 2 GHomas cell cultures available (Table 3). In ACTHoma cells, combined incubation with MF and octreotide did not significantly impact the $[Ca^{2+}]_i$ reduction elicited by MF or octreotide alone (28.9% vs. 20% or 23.9%, respectively). In GH-secreting cells, MF did not elicit changes in calcium dynamics as mentioned above and octreotide slightly reduced calcium levels in 10% of the cells analyzed. Interestingly, the co-administration of MF and octreotide produced a higher reduction of $[Ca^{2+}]_i$ (37.6% vs. 0% or 21.6%) and an increase of responsive cells compared to MF or octreotide administered alone (27.9% vs. 0% or 10%).

To better understand the effects observed in response to biguanides, we explored several signaling pathways in GHomas (Fig-5). Our results show that a short-term incubation with BF and PF numerically increase phosphorylation levels of AMPKα as compared to vehicle-treated controls, although this difference did not reach statistical significance. In contrast, MF did not alter the phosphorylation levels of AMPKα, which is in accordance with the results obtained at mRNA levels in GHomas. We also measured other signaling pathways intimately related with proliferation and survival in tumor pathologies, including PitNETs (35, 36). Thus, we observed an increase in phosphorylation levels of Akt and ERK1/2 in response to BF and PF, being this increase only significant in ERK1/2 in response to BF. On the other hand, MF did not alter phosphorylation levels of Akt or ERK1/2 (Fig-5).

DISCUSSION

PitNETs are commonly considered benign tumors due to their frequent slow growth and moderate proliferative capacity (37). However, the incidence of PitNETs is increasing, also owing to the enhanced diagnostic capacity of novel imaging techniques and improved diagnostic technologies, which have increased the sensitivity to detect pituitary neoplasms (38). Likewise, the severe morbimortality associated to PitNETs is nowadays clearly established, thereby reinforcing the necessity to develop novel therapeutic options, especially for invasive, recurrent and/or functioning tumors. In this context, there is an emerging interest in biguanides, particularly in MF, by their potential as antitumoral compounds, related to their beneficial effects in increasing insulin sensitivity and decreasing oxidative phosphorylation (39). In line with this, we recently described that different biguanides exert direct actions in the pituitary cells of two non-human primate species suggesting that the well-known metabolic effects of biguanides might be, at least in part, influenced by their actions at the pituitary level (24). However, the antitumoral actions of these compounds on different human PitNETs are poorly understood. Thus, we evaluated the direct antitumoral

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effects of different biguanides on primary cultures of functioning and non-functioning PitNETs and explored their possible underlying mechanisms.

The antiproliferative capacity of MF has been described previously in different endocrine-cancer settings, including neuroendocrine tumor cells (20, 40-42), and in various animal models (42), and is currently being tested as adjuvant therapy in several clinical trials (43). However, MF does not alter epithelial proliferation in Barrett's esophagus (44), suggesting that this drug might exert cell-and tissue-type dependent antiproliferative effects. In our study, ACTHoma cells were more sensitive to MF compared to GHoma and NFPA cells. Moreover, PF was the most effective compound in reducing cell viability in all cases, which is in accordance with a recent report from our group in neuroendocrine tumors (20). Additionally, results reporting a marked reduction of cell viability in response to MF in AtT-20 (27) and GH3 cells (26) are consistent with our results in these cell lines. However, our present findings in GHomas are not totally in line with previous reports, where MF reduced cell viability in 7 out of 8 somatotropinomas (26). These differences could be due to disparities in the experimental design, or in the basal characteristics of the GHomas analyzed. Nevertheless, although we did not observe significant changes on cell viability, there was a significant increase of apoptotic rate in response to MF in GHomas after 24h of incubation, which is consistent with results reported in GH3 cells (26). These results might be seen contradictory since the vast majority of reports seem to associate cell proliferation with apoptosis through the modulation of common and/or distinct proteins (45, 46). However, several studies have showed an imbalance between cell survival and apoptosis in pathological conditions such as breast or lung cancer (47-49). In line with the results observed in GHomas, a similar pattern of response to biguanides in terms of cell viability was found in PRLomas, which could be due to the common developmental lineage of these cells (4). However, these results differ from the growth reduction observed in response to MF in the lactotrophic MMQ cell line (50), supporting again the idea that the effect of biguanides is highly cell-type dependent.

As previously mentioned, SSAs represent important tools in the medical treatment of some PitNET types, especially in GH-secreting tumors, in relapsed or persistent disease (51); however, these treatments are in many cases ineffective (52, 53), and *in vitro* data suggests a mild effect on cell proliferation, especially when first-generation SSAs have been evaluated (54). For these reasons, the search for new pharmacological alternatives to control tumor growth and/or hormone secretion has been crucial over the last years. In this context, treatment with SSAs combined with other pharmacological therapies, such as dopamine agonists or pegvisomant, are frequently used to control hormone-related and/or other symptoms in PitNETs (55, 56). Here, we tested the combined therapy of MF with SSAs but found that this combination did not alter the inhibitory effect of MF or SSAs alone in functioning PitNETs (GH-/ACTH-omas). Similar results have been reported using AICAR (AMP mimetic compound 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK activator) and somatostatin-14, where only 1 out of 8 GHomas showed an additive effect of both compounds (22). Of note, however, our study indicate that the combination therapy of MF and SSAs seems to have a stronger effect in reducing cell viability in NFPA cell cultures, a tumor type where SSAs have shown poor efficacy (57). Although more experiments are necessary to confirm and understand this interesting observation, we might speculate that this additive effect of MF and SSAs could represent a potential therapeutic combination for patients with NFPAs since these tumors are the most resistant PitNETs to the therapeutic options currently available (29, 58).

With regard to hormone secretion, previous reports have described a time- and dose-dependent effect of MF on ACTH and GH secretion in AtT-20 and GH3 cell line (26, 27). In our study, BF and PF, but not MF treatment clearly reduced GH and PRL secretion without

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altering ACTH release. These results are in part comparable to a previous recent report from our group performed in neuroendocrine tumor cells, in which MF had no effect on hormone secretion in BON-1 or QGP-1 cell cultures, whereas PF decreased hormone secretion in BON-1, but not in QGP-1 cell cultures (20). However, we found that all biguanides significantly reduced GH secretion in the GH3 cell line, supporting again the notion of a cell-type specific effect of different biguanides on pituitary cells. In line with the results observed in cell viability, the combination therapy with SSAs did not increase the effectiveness of the SSA in monotherapy at the level of hormone secretion. Indeed, the fact that MF did not impact on SSAs effect at both, cell viability and hormone secretion, levels might suggest shared mechanisms of action between MF and SSAs in pituitary tumor cells.

In contrast with the results published recently by our group in normal pituitary glands from non-human primate species (24), MF and BF did not modify GH, POMC or SSTs at mRNA expression levels. On the contrary, PF significantly increased GH expression levels, which could occur as a feedback loop mechanism in order to compensate the striking reduction of GH secretion observed in GHomas and GH3 cell line in response to this compound. PF also increased SST_2 and SST_5 expression levels in ACTH- and GH-secreting cells, which is in line with the similar increase observed in normal pituitary gland in *Papio anubis* (24). These data indicate that the actions of different biguanide types in PitNET cells are not only confined to the regulation of cell viability and/or hormonal secretions, but also included the regulation of the synthesis of key genes involved in the regulation of pituitary pathophysiology (i.e. pituitary hormones and SSTs). Although additional experimental work is necessary to confirm, understand and achieve a definitive interpretation of the biological meaning of these results, our data adds compelling evidence on the direct effects that biguanides exert on the expression profile of relevant pituitary genes, which might pave the way towards the identification and validation of additional biomarkers and/or therapeutic targets in these pathologies.

Our report also provides information about the signaling pathways underlying the effects of biguanides in PitNET cells. Calcium is a relevant second messenger for pituitary cell physiology which has been classically associated to pituitary hormone secretion (59). In this sense, our results showed an inhibitory action on $[Ca^{2+}]_i$ levels predominantly in ACTHomas and NFPAs, and not in GHomas or PRLomas. Intriguingly, the calcium dynamics observed in our study do not seem to be associated to hormone secretion, since biguanides did not modify ACTH secretion from corticotropinoma cells. Therefore, our results suggest that calcium kinetics in response to biguanides could be more related with another functional parameter such as cell viability (60, 61). Additionally, combination therapy of MF with SSAs did not alter the effect of MF as monotherapy in ACTHomas, but seemed to increase the effect of octreotide in GHomas, although this increase was not enough to associate it to any functional endpoint. Furthermore, as expected, we also found a regulation on the phosphorylation levels of AMPK (considered the central mediator of MF effects in different tissues/organs (34) in response to BF and PF (which is in line with our results at mRNA expression levels). However, this increase in AMPK phosphorylation levels was not observed in response to MF, which is in contrast with the increase reported in GH3 and AtT-20 cell lines (25-27). In the same line, in this study we found an overall increase on phosphorylation levels of Akt and ERK1/2 in response of BF and PF, but not MF. It should be mentioned that an increase in the phosphorylation levels of these two pathways has been related in several reports with a reduction of cell proliferation in response to short-period of incubations with SSAs or dopamine analogues in PitNETs (62, 63). Therefore, our findings demonstrate that some biguanides act through signaling pathways that has not been linked to AMPK, suggesting that some actions of these compounds could be exerted though AMPK-independent mechanisms in PitNET cells.

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In conclusion, our study provides primary evidence that biguanides exert important anti-proliferative and anti-secretory effects in some PitNET cell types through the modulation of AMPK-dependent ($[Ca^{2+}]_i$ dynamics, PI3K-Akt pathway) and independent (ERK pathway) mechanisms. Moreover, combination of MF and SSAs treatment did not exert additional antitumoral effects in functioning PitNETs, which suggest shared mechanisms of actions. Of note, combined therapy with MF and SSAs might represent a potential new therapeutic approach for patients with NFPAs. Taken together, our results unveil a clear overall antitumoral effect of different biguanides on PitNET cells and pave the way to consider these compounds as a potential new option in the treatment of these severe pathologies.

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The authors have nothing to disclose.

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Figure 1: Dose-response experiment of cell viability in response to metformin (5 and 10 mM) in primary ACTHoma (n=7), GHoma (n=3) and NFPA (n=7) cell cultures, measured by Alamar-blue reduction. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* $p<0.05$) indicate statistically significant differences.

Figure 2: Measurement of cell viability (24-72 h) and apoptosis in response to different biguanides alone or in response to the combination of metformin with somatostatin analogues (octreotide or pasireotide) in primary PitNET cell cultures and pituitary cell lines. (A) Effect of metformin (MF; 10 mM), buformin (BF; 5 mM) and phenformin (PF; 5 mM) on cell viability in primary ACTHoma cell cultures (MF: n=10; BF: n=6; PF: n=5) and in the corticotropinoma AtT-20 cell line (MF: n=9; BF: n=6; PF: n=7), measured by Alamar-blue reduction. (B) Effect of MF alone or in combination with octreotide or pasireotide on cell viability in ACTHomas (n=3). (C) Effect of MF, BF and PF in primary GHoma cell cultures (MF: n=9; BF and PF: n=4) and in the somatotropinoma GH3 cell line (n=4). (D) Effect of MF alone or in combination with octreotide or pasireotide on cell viability in GHomas (n=5). (E) Effect of biguanides in primary NFPA cell cultures (MF: n=11; BF: n=7; PF: n=8). (F) Effect of MF alone or in combination with octreotide or pasireotide on cell viability in NFPAs (n=2). (G) Effect of biguanides in primary PRLoma cell cultures (n=3). (H) Effect of MF on apoptosis (24h treatment) in primary GHoma cell cultures measured by Caspase-Glo 3/7 assay. Data are expressed as percent of vehicle-treated controls (set at

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100%) within experiment. Values represent the mean ± SEM. Asterisks (* p<0.05; ** p<0.01; *** p<0.001) indicate statistically significant differences. In cases where less than three experiments were performed, no significance tests were performed.

Figure 3: Hormone secretion in response to different biguanides alone or to metformin in combination with octreotide or pasireotide in human PitNET cell cultures and cell lines. (A) Effect of metformin (MF; 10 mM). buformin (BF; 5 mM) and phenformin (PF; 5 mM) on ACTH secretion in primary ACTHoma cell cultures (24h treatment; MF: n=6; BF and PF: n=3), determined by commercial ELISA kit. (B) Effect of MF, BF and PF on GH secretion in primary GHoma cell cultures (MF: n=8; BF: n=4; PF: n=3). (C) Effect of MF alone or in combination with octreotide or pasireotide on GH secretion in primary GHoma cell cultures (n=4). (D) Effect of biguanides on PRL secretion in primary PRLoma cell cultures (n=2). (E) Effect of MF on GH secretion in GH3 cell line (n=5), determined by commercial ELISA kit. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* p<0.05; ** p<0.01; *** p<0.001) indicate statistically significant differences. In cases where less than three experiments were performed, no significance tests were performed.

Figure 4: Measurement of mRNA expression levels of key genes in response to different biguanides in human PitNET cell cultures and cell lines. (A-B, E) Effect of metformin (MF; 10 mM). buformin (BF; 5 mM) and phenformin (PF; 5 mM) on the expression levels of different genes in primary ACTHoma cell cultures (MF: n=3; BF and PF: n=2) and in the corticotropinoma AtT-20 cell line (n=4). (C-D, F) Effect of MF, BF and PF on expression levels in primary GHoma cell cultures (n=4) and in the somatotropinoma GH3 cell line (n=4). Expression levels were measured by quantitative-PCR and adjusted by normalization factor (NF). Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* p<0.05; ** p<0.01; *** p<0.001) indicate statistically significant differences. In cases where less than three experiments were performed, no significance tests were performed.

Figure 5: Measurement of phosphorylation levels of AMPK, Akt and ERK1/2 in response to different biguanides in primary GHoma cell cultures. Representative Western Blots and quantification of levels of p-AMPK/total AMPK (n=3), p-Akt/total Akt (n=2) and p-ERK1/2/ total ERK1/2 (n=3) in response to MF (10 mM), BF (5 mM) and PF (5 mM) in GHomas. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* p<0.05) indicate statistically significant differences. In cases where less than three experiments were performed, no significance tests were performed.

Table 1: Demographic data of patients included in the study.

Tumor type	N	Sex (% of women)	Age (min-max)
Corticotropinomas	13	92%	56 (18-79)
Somatotropinomas	13	54%	50 (29-64)
Prolactinomas	3	33%	25 (20-34)
Non-functioning pituitary adenomas	13	62%	49 (24-75)

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Table 2: Results from free cytosolic calcium dynamic assays in PitNET cells in response to biguanides.

Treatment	# samples	Cells analyzed	% PRC	% PRM ± SEM	Time (s) ± SEM
ACTHomas					
Metformin	3/5	236	9.80%	23.34 ± 1.77	49.38 ± 2.67
Buformin	3/4	154	46.80%	25.5 ± 1.64	61.55 ± 3.10
Phenformin	2/3	119	30.25%	27.11 ± 2.14	65.98 ± 2.78
GHomas					
Metformin	0/3	150	0	-	-
Buformin	0/3	79	0	-	-
Phenformin	0/3	81	0	-	-
NFPAs					
Metformin	2/4	144	16.00%	30.62 ± 2.62	54.7 ± 5.67
Buformin	2/3	106	18.90%	20.6 ± 3.2	54.7 ± 3.46
Phenformin	2/3	105	32.40%	44.7 ± 4.92	62.2 ± 2.2
PRLomas					
Metformin	1/2	60	6.70%	18.4 ± 0.92	61.25 ± 2.07
Buformin	0/2	49	0	-	-
Phenformin	0/2	58	0	-	-

samples: number of responsive samples of the total of samples analyzed.
Cells analyzed: total of individual cells analyzed.
% PRC: percentage of responsive cells in responsive samples.
Time: time of response to biguanides administration.

Table 3: Results from free cytosolic calcium dynamic assays in PitNET cells in response to metformin alone or in combination with octreotide.

Treatment	# samples	Cells analyzed	% PRC	% PRM ± SEM	Time (s) ± SEM
ACTHomas					
Metformin	1/2	58	12.1%	20 ± 1.77	51.43 ± 1.95
Octreotide	1/2	67	4.5%	23.9 ± 3.62	110 ± 0
MF + Octreotide	1/2	66	4.5%	28.9 ± 5.44	45 ± 4.08
GHomas					
Metformin	0/2	70	0	-	-
Octreotide	2/2	70	10%	21.6 ± 0.8	89.4 ± 3.71
MF + Octreotide	1/2	68	27.9%	37.6 ± 3.89	85 ± 0

samples: number of responsive samples of the total of samples analyzed.
Cells analyzed: total of individual cells analyzed.
% PRC: percentage of responsive cells in responsive samples.
Time: time of response to biguanides administration.

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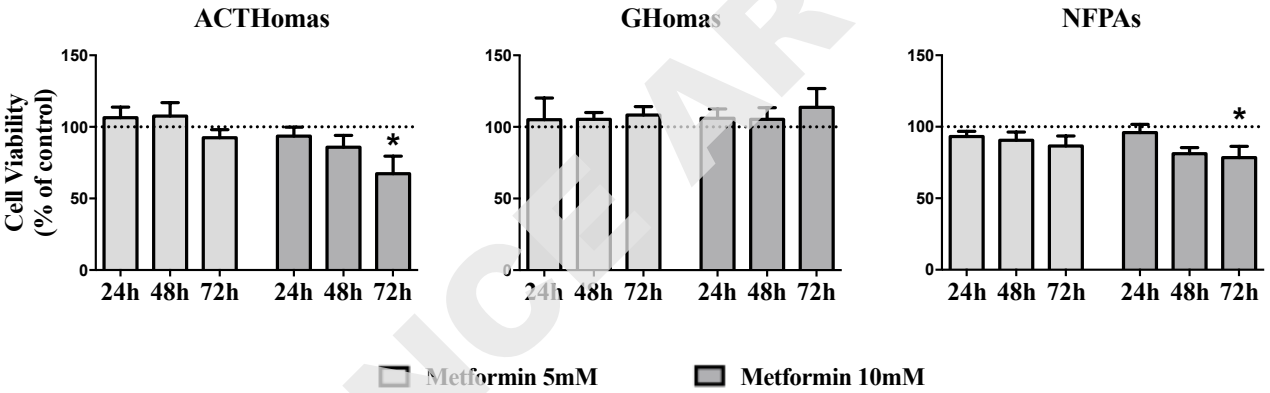

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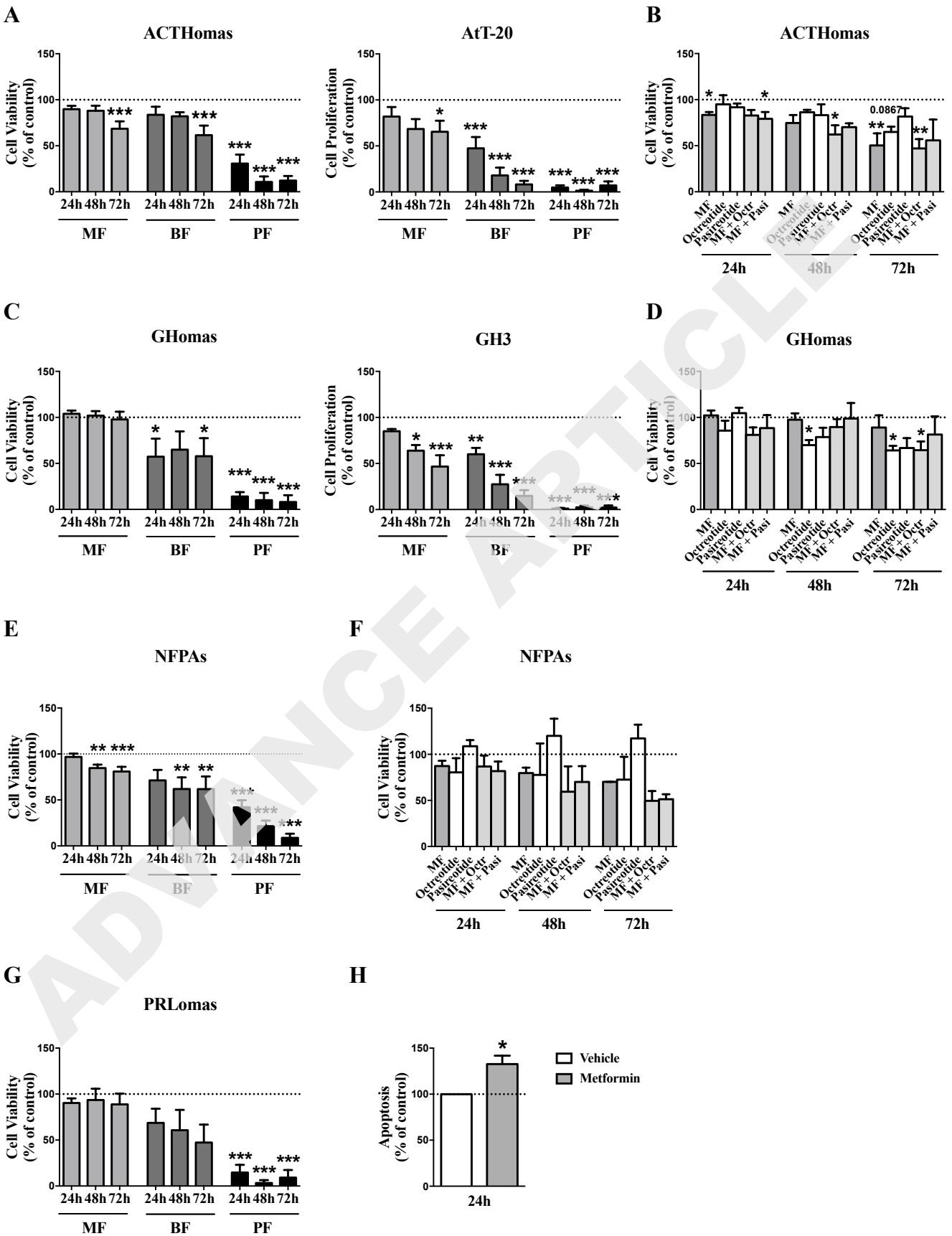

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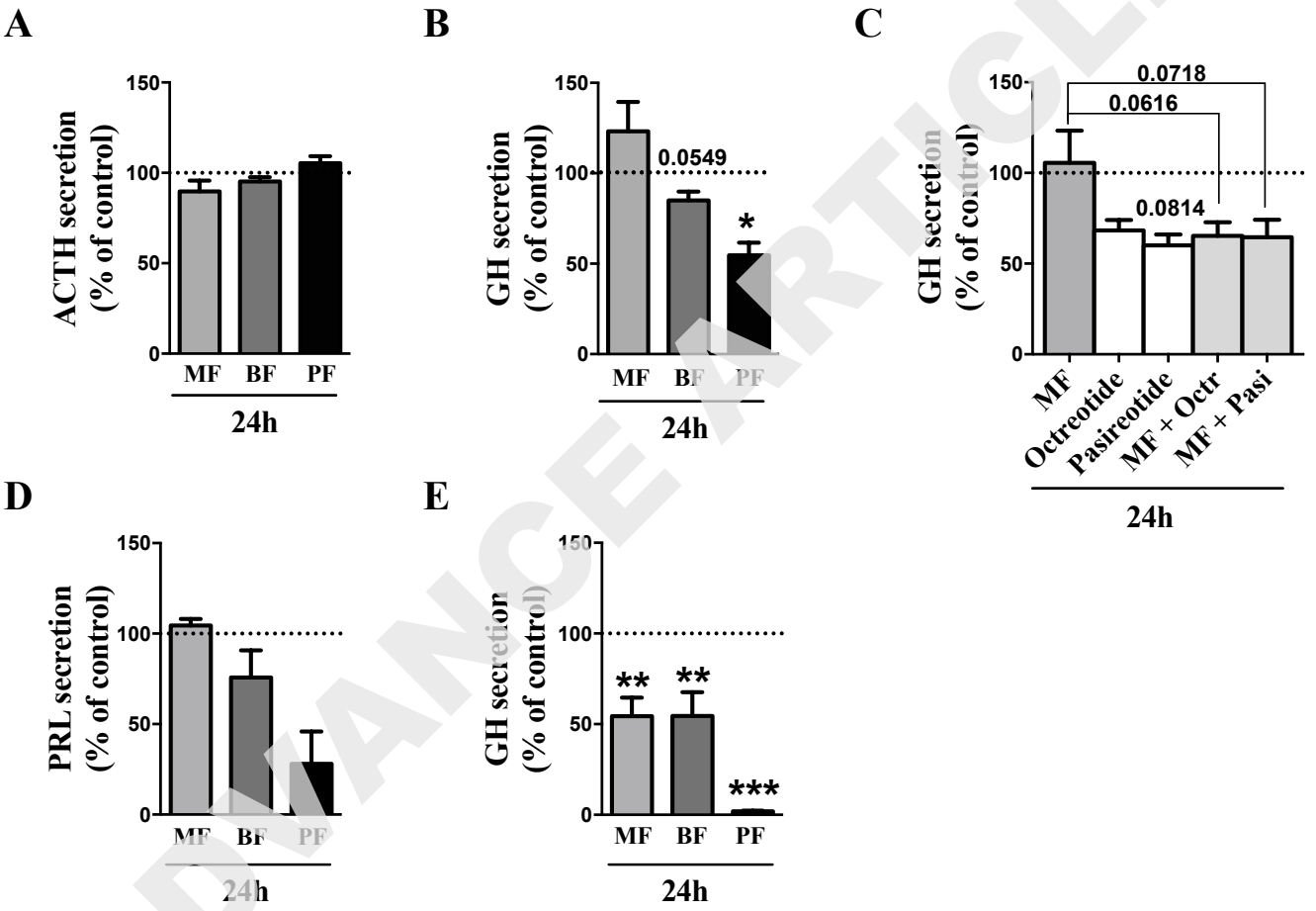

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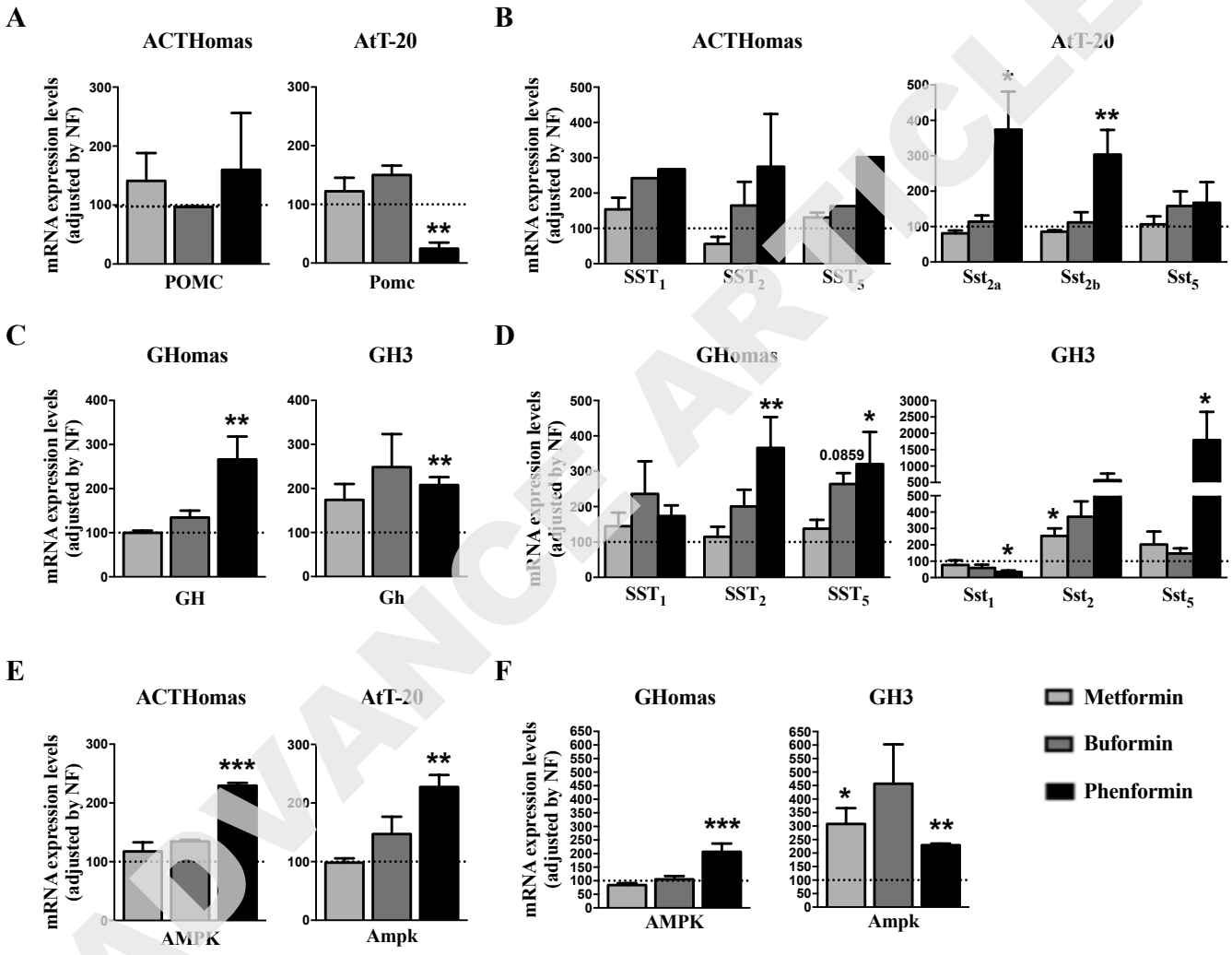

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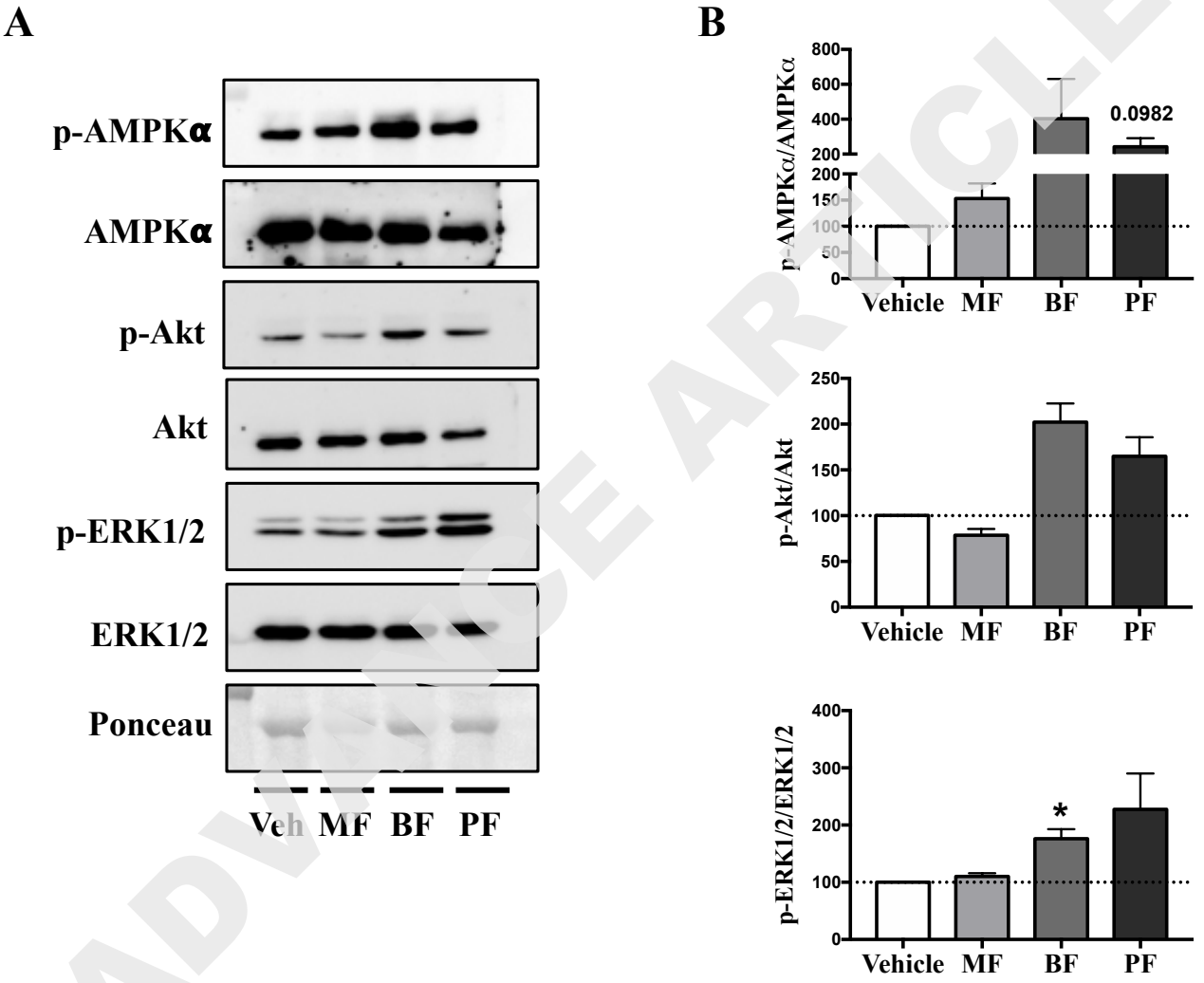

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Table 4: General characteristics of patients with different types of PitNETs treated or not with metformin.

	ACTHomas				GHomas				NFPTs			
General characteristics	**Total (42)**	**Patients with metformin (8)**	**Patients without metformin (34)**	***p*[a]**	**Total (28)**	**Patients with metformin (3)**	**Patients without metformin (25)**	***p*[a]**	**Total (62)**	**Patients with metformin (7)**	**Patients without metformin (55)**	***p*[a]**
Sex				ns				ns	54.8 (34/62)	42.9 (3/7)	56.4 (31/55)	ns
Male	4.8 (2/42)	12.5 (1/8)	5.9 (2/34)		39.3 (11/28)	33.3 (1/3)	44.0 (11/25)					
Female	95.2 (40/42)	87.5 (7/8)	94.1 (32/34)		60.7 (17/28)	66.6 (2/3)	68.0 (17/25)					
Age (years)	45 ± 2.07	48 ± 4,81	44.3 ± 2.3	ns	44 ± 3.13	63 ± 10.5	43 ± 3.2	ns	56 ± 13.81	64 ± 2.31	55 ± 1.91	ns
BMI (Kg/m^2)	44.9 ± 12.1 (35/42)	31.4 ± 2.5 (6/8)	47.7 ± 14.8 (29/34)	ns	28.76 ± 1.06 (15/28)	26.53 ± 0.0 (1/3)	28,9 ± 1.1 (14/25)	ns	28.1 ± 4.62 (21/62)	29.3 ± 0.90 (3/7)	27.9 ± 1,17 (18/55)	ns
Lesion type				ns				ns				ns
Macroadenoma	45.2 (19/42)	62.5 (5/8)	41.2 (14/34)		95.8 (23/24)	100 (2/2)	95.5 (21/22)		100.0 (60/60)	100.0 (7/7)	100.0 (7/7)	
Microadenoma	42.9 (18/42)	25 (2/8)	47.0 (16/34)		4.2 (1/24)	0 (0/3)	4.5 (1/22)		0.0 (0/60)	0.0 (0/7)	0.0 (0/7)	
Not visible at MRI	11.9 (5/42)	12.5 (1/8)	11.8 (4/34)		0.0 (0/24)	0 (0/3)	0 (0/22)		0.0 (0/60)	0.0 (0/7)	0.0 (0/7)	
Tumor size (mm)	11.5 ± 1.41 (35/42)	14.4 ± 4.4 (7/8)	10.5 ± 1.4 (28/34)	ns	17.9 ± 1.3 (24/28)	19.0 ± 1 (2/3)	17.8 ±1.4 (22/25)	ns	20.8 ± 9.41 (27/62)	19.3 ± 3.84 (3/7)	21.0 ± 2.31 (23/55)	ns
Cephalea	11.8 (4/34)	14.3 (1/7)	11.1 (3/27)	ns	50 (14/28)	33.3 (1/3)	52 (13/25)	ns	38.7 (24/62)	28.6 (2/7)	40.0 (22/55)	ns
Visual alterations	2.6 (1/38)	0.0 (0/8)	3.3 (1/30)	ns	16.7 (4/28)	0.0 (0/3)	16 (4/25)	ns	48.3 (28/58)	71.4 (5/7)	44.2 (23/52)	ns
Extrasellar growth	24.4 (10/41)	42.9 (3/7)	21.2 (7/33)	ns	72 (18/25)	50 (1/2)	73.9 (17/23)	ns	89.4 (42/47)	85.7 (6/7)	90.0 (36/40)	ns
Pre-surgery treatment												
Ketoconazole	86.8 (33/38)	100.0 (8/8)	83.3 (25/30)	ns	NA	NA	NA	ns	NA	NA	NA	ns
SSA	NA	NA	NA		33.3 (9/27)	66.6 (2/3)	29.2 (7/24)	ns	NA	NA	NA	ns
Cabergoline	14.3 (6/42)	37.5 (3/8)	9.1 (3/33)	ns	3.7 (1/27)	0.0 (0/3)	4.2 (1/24)	ns	9.1 (5/55)	0.0 (0/5)	10.0 (5/50)	
SSA + cabergoline	NA	NA	NA		29.6 (8/27)	33.3 (1/3)	29.2 (7/24)		NA	NA	NA	
ACTH at baseline	81.2 ± 9,5 (39/42)	108.8 ±27.7 (8/8)	74.1 ± 9.5 (31/34)	ns	-	-	-	-	-	-	-	-
GH at baseline	-	-	-	-	8.3 ± 1.52 (27/28)	8.2 ± 2.92 (3/3)	8.3 ± 1.69 (23/25)	ns	-	-	-	-
IGF1 at baseline	-	-	-	-	637.0 ± 53.36 (28/28)	578.4 ± 75.78 (3/3)	644.0 ±59.26 (25/25)	ns	-	-	-	-
Persistence after surgery	50.0 (20/40)	50.0 (4/8)	50.0 (16/32)	ns	NA	NA	NA	-	78.3 (47/60)	85.7 (6/7)	77.4 (41/53)	ns

Data given as % (nº/total) or mean ± SD, unless otherwise indicated. [a]For the comparison between patients treated with metformin with those not treated with metformin. NA: not available.

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Original Paper

The Pituitary Gland is a Novel Major Site of Action of Metformin in Non-Human Primates: a Potential Path to Expand and Integrate Its Metabolic Actions

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Key Words
Pituitary • Metformin • Phenformin • Growth hormone • Primates • Hormones

Abstract
Background/Aims: Biguanides are anti-hyperglycaemic agents used to treat diabetes by acting primarily on the liver, inhibiting hepatic gluconeogenesis. However, biguanides may target other key metabolic tissues to exert beneficial actions. As the "master endocrine gland", the pituitary is a true homeostatic sensor that controls whole body homeostasis and metabolism by integrating central and peripheral signals. However, whether the pituitary is a primary site of biguanides action in normal adult humans/primates remains unknown. Therefore, we aimed to elucidate the direct effects of two biguanides (metformin/phenformin) on the expression and secretion of all anterior pituitary hormones in two non-human primate species (*Papio anubis* and *Macaca fascicularis*), and the molecular/signalling-mechanisms behind these actions. ***Methods:*** Primary pituitary cell cultures from baboons and macaques were used to determine the direct impact of metformin/phenformin (alone and combined with primary regulators) on the functioning of all pituitary cell-types (i.e. expression/secretion/signaling-pathways, etc). ***Results:*** Metformin/phenformin inhibited basal, but not GHRH/ghrelin-stimulated GH/ACTH/FSH-secretion and GH/POMC-expression, without altering secretion or expression of other pituitary hormones (PRL/LH/TSH), FSH-expression or cell viability in both primate models. These biguanide actions are likely mediated through modulation of: 1) common (mTOR/PI3K/intracellular-Ca^{2+}mobilization) and distinct (MAPK) signaling pathways; and 2) gene expression of key receptors regulating somatotrope/corticotrope/gonadotrope function (i.e. upregulation of *SSTR2/SSTR5/INSR/IGF1R/LEPR*). ***Conclusion:*** The pituitary gland is a primary

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target of biguanide actions wherein they modulate somatotrope/corticotrope/gonadotrope-function through multiple molecular/signaling pathways in non-human primate-models. This suggests that the well-known metabolic effects of biguanides might be, at least in part, influenced by their actions at the pituitary level.

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Introduction

Biguanides are a synthetic class of antidiabetic (anti-hyperglycaemic) agents constituted by two N-linked guanidine rings (i.e. metformin and phenformin), whose origin derives from galegine (isoamylene guanidine), a natural compound found in *Galega officinalis* [1]. Metformin has been the most frequently prescribed drug used to treat type-2 diabetes (T2D) for many years, and has been found to be safe and efficacious both as monotherapy and in combination with other oral antidiabetic agents. However, the sites and mechanisms of actions of metformin have been only partially explored and remain somewhat controversial. Specifically, the liver is presumed to be the primary site of metformin function, as the antihyperglycemic effect of metformin is mainly due to the inhibition of hepatic gluconeogenesis, wherein AMP-activated protein kinase (AMPK), a protein kinase that plays a key role in maintaining energy homeostasis, is assumed to be the prime hepatic target of metformin [2, 3]. However, recent studies have revealed that metformin, besides its glucose-lowering action, might exert additional, promising actions for the modulation of whole body homeostasis and metabolism, by specifically targeting other key endocrine/metabolic tissues, as well as by exerting other positive effects through additional mechanisms of actions (i.e. beneficial use in the treatment of cancer, cardiovascular diseases, aging, immunity and polycystic ovarian syndrome, etc.); although, these and other effects and mechanism of actions of metformin require further investigation [4-12]. Actually, it is still unclear if AMPK is the central mediator of metformin effects in all metabolic tissues and, therefore, the existence of both AMPK-dependent and independent mechanisms has been proposed [2, 13, 14].

In this context, the pituitary gland, classically known as the "master endocrine gland", is currently considered also a true metabolic sensor for whole body function, and one of the most important players in the control of body homeostasis, integrating central and peripheral signals [15]. Specifically, the five hormone-producing cells of the adenohypophysis (i.e. GH-producing somatotropes, PRL-producing lactotropes, ACTH-producing corticotropes, TSH-producing thyrotropes, and FSH/LH-producing gonadotropes) receive multiple central and peripheral signals and, the integration of these signals results in the modulation of the corresponding hormonal secretions, which, in turn, control key peripheral organs and tissues related with essential metabolic functions such as growth, lactation, stress responses, appetite, reproduction, whole-body metabolism and puberty [16]. Inasmuch as metformin is involved in the modulation of a wide variety of metabolic processes in humans, it seems reasonable to think that this agent could exert some of these effects by directly influencing pituitary gland function. Indeed, some observations suggest that metformin is able to modify pituitary hormone levels in some pathological conditions (i.e. patients with pituitary adenomas, hypothyroidism or polycystic ovary syndrome (PCOS) [17, 18]), although these results are scattered, inconsistent and controversial.

To the best of our knowledge, no studies have explored hitherto, on suitable models, how metformin or other biguanides agents can modulate directly the function of all the anterior pituitary cell types in normal adult humans or in primate species, and what intracellular signaling pathways would be involved in these putative actions. Accordingly, in the present study, we aimed at determining, for the first time, the direct effects of metformin and phenformin on the expression and secretion of all anterior pituitary hormones in two primate model species that closely resemble human physiology: *Papio anubis* (baboons) and *Macaca fascicularis* [19-21]. In addition, we also used primary pituitary cell cultures from baboons to better understand the mechanisms behind these actions, by evaluating the effects of these biguanides on the expression of selected key receptors and transcriptional factors involved in the normal functioning of the pituitary cell types, and by assessing the precise contribution of different signaling pathways in the actions of metformin using standard pharmacological (inhibitory) approaches.

Materials and Methods

Reagents

All reagents and inhibitors of intracellular signaling pathways used in this study were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Ghrelin was purchased from Phoenix Pharmaceuticals (Burlingame, CA). α-MEM, HEPES, horse serum, and penicillin-streptomycin were obtained from Invitrogen (Grand Island, NY), and U73122 was purchased from Cayman Chemical (Ann Arbor, MI).

Animals and tissue collection

Pituitary glands were obtained from randomly cyclic female baboons (Olive Baboon; *Papio anubis*; n=6; 7–12 years of age) and macaques (*Macaca fascicularis*; n=3; 7 years of age) within 15 min after sodium pentobarbital overdose as previously reported [22, 23]. The animals represent control animals from a breeding colony at the University of Illinois at Chicago (UIC). All procedures were approved and conducted under the Institutional Animal Care and Use Committee at the UIC (Chicago, IL), and all methods were carried out in accordance with relevant guidelines and regulations. Right after the animals were euthanized, pituitaries were excised, placed in sterile cold (4 °C) basic media (α-MEM with: 0.15% BSA, 6nM HEPES, 10-IU/mL penicillin, and 10-μg/mL streptomycin) and immediately transported to the laboratory in sterile conditions.

Primary pituitary cell cultures

Pars distalis of the pituitary was isolated, washed twice in fresh media, and cut into small pieces (~20 – 40 mg) with surgical blades; then, fragments were incubated in 30 mL S-MEM medium complemented with 0.3% trypsin (Beckton, Dickinson and Company, Sparks, MD, USA) in a spinner flask (Bellco Glass, Vineland, NJ, USA) for 2 h at 37 °C under gentle shaking to obtain dispersed single cells for culture, as previously reported [22-25]. To avoid fibroblast contamination, suspensions of dispersed pituitary cells were filtered through a nylon gauze of 130 μM-mesh and cultured in media with D-Valine (replaced for L-valine) to selectively inhibit fibroblast proliferation/overgrowth. In addition, visual inspection of primary cell cultures at the time of experimental assays showed no sign of cells displaying the typical fibroblast-like morphology.

Single cells (50.000-200.000 cells/ well) were plated onto 48- or 24-well plates in media containing 10% fetal horse serum. After 36h incubation (37°C, 5% CO_2), media was removed and cells pre-incubated for 1h with fresh, warm (37 °C) serum-free medium to stabilize basal hormone secretion. After this pre-incubation period, cells were incubated with serum-free medium alone (controls) or serum-free media containing the following treatments: *1)* metformin or phenformin alone (10^{-7} to $5x10^{-3}$ M; *dose-response experiment*; 4h incubation; doses selected based on previous studies [12, 26, 27]); *2)* metformin or phenformin alone ($5x10^{-3}$ M) for 4h and 24h (*time-course experiment*); *3)* metformin or phenformin alone ($5x10^{-3}$ M) or in combination with GH-releasing hormone (GHRH; 10 nM), acylated-ghrelin (10 nM) or gonadotropin-releasing hormone (GnRH, 10 nM) for 4h. Cells from different pituitaries (i.e. n=6 from baboons and n=3 from macaques) were not pooled. It should be noted that, given the limited source of macaque cell preparations (n=3), and the amount of cells obtained after dispersion of the pituitary glands, we were not able to reproduce the total amount of experiments included herein in both primate species.

To study the intracellular signaling pathways involved in the metformin-mediated actions on baboon pituitary hormone release, after the 1h pre-incubation period with serum-free media, cells were incubated for an additional 90-minute period in serum-free media containing the following inhibitors of selected intracellular signaling pathways: mammalian target of rapamycin (mTOR; rapamycin; 10 μM), phosphatidylinositol 3-kinase activity (PI3K; wortmannin; 1 μM), mitogen-activated protein kinase activity (MAPK; PD-98, 059; 10μM), extracellular Ca^{2+} L-type channels (nifedipine; 1μM), intracellular Ca^{2+} channels (thapsigargin; 10μM), adenylyl cyclase (AC; MDL-12, 330A; 10μM), and phospholipase-C (PLC; U73122; 50μM). Thereafter, the media were replaced with media with the specific inhibitor alone (vehicle) or media with the inhibitor containing metformin ($5x10^{-3}$ M), and cells were incubated for an additional 4h. Additional controls consisted of serum-free media alone or media with metformin (in all cases without inhibitors). Doses for GHRH, acylated-ghrelin, GnRH or inhibitors of intracellular signaling pathways were selected based on previous studies [22-25, 28, 29]. At the end of the corresponding incubation periods with the different treatments, media were collected for hormone analysis using commercial ELISAs (see section below) and, in selected cases, cells were processed for total RNA recovery and assessment of mRNA levels by quantitative real-time PCR (qPCR; see section below).

Hormone release analysis.

GH, PRL, ACTH, LH, FSH and TSH hormone concentrations in the culture media were measured using human commercial ELISAs [Human: GH, PRL, ACTH, FSH, LH and TSH (reference numbers: EIA-1787, EIA-

1291, EIA-3647, EIA-1288, EIA-1289 and EIA-1790, respectively; DRG, Mountainside, NJ)], as previously described [22, 24]. All the assays were performed following the manufacturer's instructions where the information regarding specificity, detectability and reproducibility for each of the assays can be accessed at the websites of the indicated company.

RNA isolation, reverse transcription and qPCR of primate transcripts

Primary pituitary cell cultures from baboons were processed for recovery of total RNA and the subsequent quantification of the amount of RNA recovered using kits and methods previously described [23, 29]. Briefly, total RNA was extracted using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA) with deoxyribonuclease treatment. The amount of RNA recovered was quantified by the Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR) and reverse transcribed in a 20µl volume using random-hexamer primers and the cDNA First Strand Synthesis kit (MRI Fermentas, Hanover, MD). cDNAs were treated with ribonuclease H (1 U; MRI Fermentas) and amplified by qPCR using a Stratagene Mx3000p real-time PCR machine and the Brilliant III Ultra-Fast SYBR® QPCR Master Mix (Stratagene, La Jolla, CA, USA). To estimate mRNA copy number, samples were run against specific synthetic standards (1-10^6 copies of synthetic cDNA template for each transcript of interest) run on the same plate. Details regarding the development, validation, and application of a qPCR as well as the specific sets of primers sequences to measure expression levels of primates transcripts included in this study, including cyclophilin A (PPIA; used as a reference, housekeeping gene), have been reported previously [22-24, 29, 30]. New baboon primer sequences were used in the present study to amplify *GNRHR,* (sense, TGCCTCTTCATCATCCCTCTT and antisense AGTCTTCAGCCGTGCTCTTG; accession number, NM000406; product size, 144 pb) and *LEPR* (sense, GGAAGGAGTGGGAAAACCAAAG and antisense, CCAAGCAATAAGATGGAAGAGG; accession number, XM_009210050.2; product size, 126 pb). To control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, mRNA copy numbers of the baboon transcripts analyzed were adjusted by cyclophilin-A expression, where baboon cyclophilin-A mRNA levels did not significantly vary between experimental groups (data not shown).

Cell viability

In order to determine if metformin or phenformin altered cell viability, trypan blue (Sigma, St. Louis, MO) assay was used as previously reported [22], following the manufacturer's instructions. Specifically, macaque and baboon primary pituitary cell cultures were plated onto 48-well tissue culture plates (100, 000 cells/well: 3 wells/treatment) in basic medium containing 10% horse serum. After 24h of incubation (37°C, 5% CO_2), medium was removed, and cells were preincubated for 4h in fresh, warm (37°C) serum-free medium to induce cells synchronization. Then, cells were washed 3 times with serum-free medium and incubated 24h with serum-free medium alone (controls) or containing metformin or phenformin ($5x10^{-3}$ M). After that, cell viability was evaluated using trypan blue reagent (counting a minimum of 300 cells/well/treatment).

Statistical analysis

Samples from all groups within an experiment were processed at the same time. Results are expressed as mean ± SEM and were obtained from at least three separate, independent experiments carried out on different days, and with different cell preparations (3-4 replicate culture wells/treatment/experiment). To normalize values within each treatment and minimize intragroup variations in the different experiments (i.e. different age of the donor, metabolic environment, stage of the estrus cycle, etc.), the values obtained were compared with the corresponding vehicle-treated controls (set at 100%), where this style of data presentation does not alter the relative differences between the different biguanides-treated and vehicle-treated groups. Differences between experimental groups were assessed by one-way ANOVA [or two-way ANOVA when the intracellular signaling pathways, with treatments with and without (controls) specific inhibitors, were studied] followed by Fisher's test for multiple comparisons. $P<0.05$ was considered significant difference. All statistical analyses were performed using GB-STAT software package (Dynamic Microsystems, Inc., Silver Spring, MD).

Results

Direct effects of metformin and phenformin on primate pituitary hormone release.

Incubation of cultured baboon and macaque pituitary cells with increasing doses of metformin (from 10^{-7} to $5x10^{-3}$ M) for 4h revealed significant inhibitory effects on GH, ACTH and FSH release in a concentration-dependent manner (at doses equal to or above

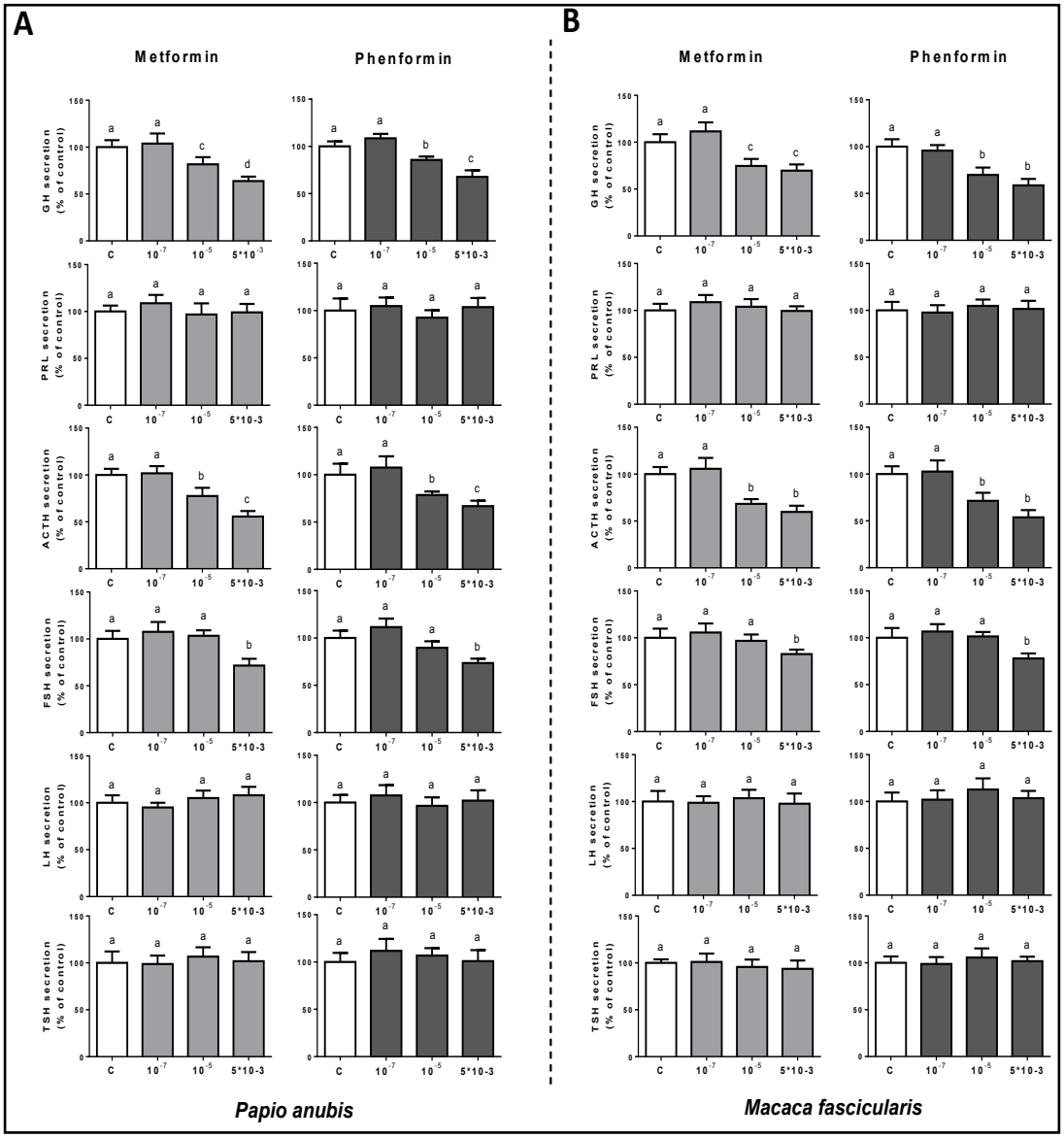


Fig. 1. Dose-response (4h) of metformin and phenformin (5mM, 10^{-5}M and 10^{-7}M) on the secretion of GH, PRL, ACTH, FSH, LH and TSH in primary pituitary cell cultures from baboons (A: metformin, n=4; phenformin, n=1) and macaques (B; n=3). Data are expressed as percent of control (set at 100%) and represent the mean ± SEM (n=3-4 wells/experiments). Values that do not share a common letter (a, b and c) are statistically different.

10^{-5} M for GH/ACTH and $5x10^{-3}$ M for FSH; Fig. 1A-B; n=3-4). Conversely, metformin failed to alter basal PRL, LH or TSH release at all the doses tested (Fig. 1A-B). Treatment with phenformin exerted the same effects than metformin on the secretion of all the pituitary hormones [Fig. 1B; macaque-model (n=3), and a single pilot experiment performed in the baboon-model (Fig. 1A)]. Based on these results, the dose of metformin and phenformin that caused a maximal or significant decrease of GH, ACTH and TSH release, $5x10^{-3}$ M, was chosen to further analyze the action of these agents on primate pituitary function.

Direct effects of metformin and phenformin after a short vs. long incubation period on primate pituitary hormone release

In a separate experiment, treatment with $5x10^{-3}$ M metformin and phenformin, for different incubation times, i.e. short- (4h) and long-term (24h), confirmed an inhibitory effect

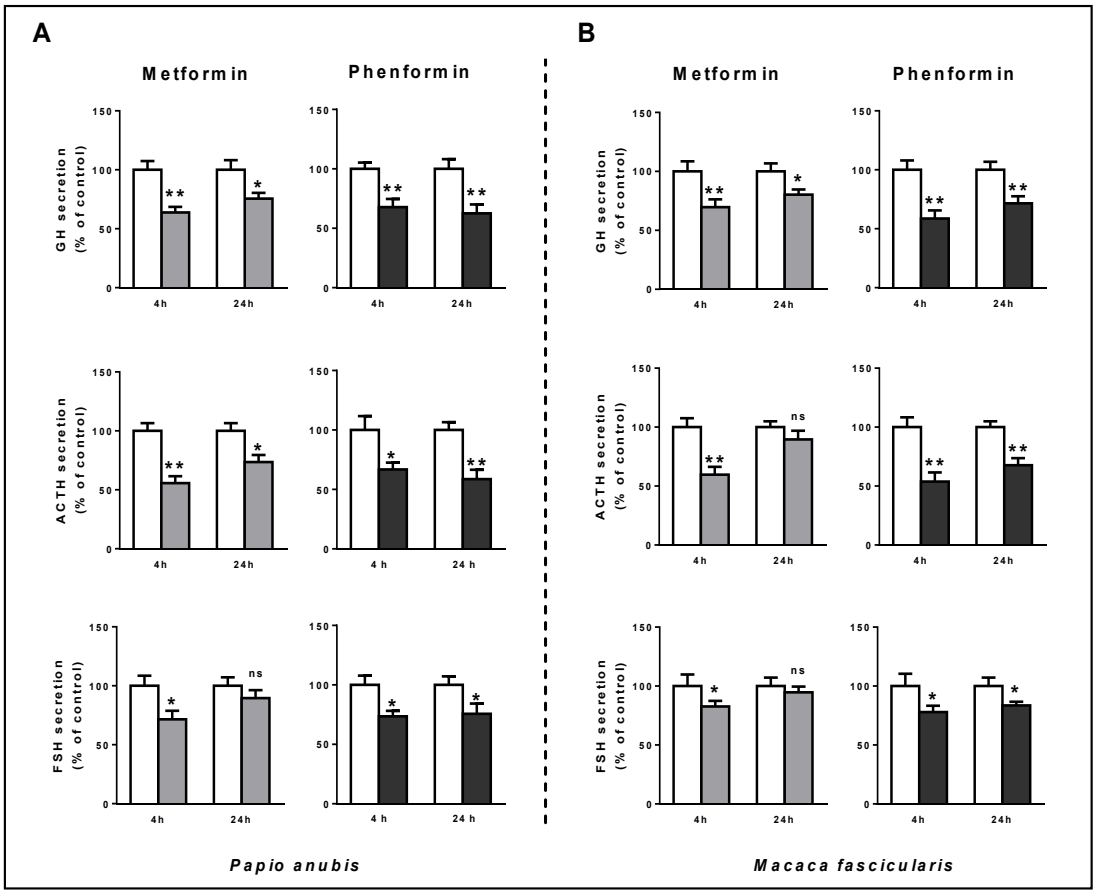


Fig. 2. Time response (4h and 24h) of metformin and phenformin (5 mM) on GH, ACTH and FSH secretion in primary pituitary cell cultures from baboons (A: metformin, n=3-4; phenformin, n=1) and macaques (B; n=3). Data are expressed as percent of control (set at 100%) and represent the mean ± SEM (n=3-4 wells/ experiments). Asterisks (*p<0.05, **p<0.01) indicate values that significantly differ from their respective control values.

of both biguanides on GH, ACTH and FSH release [Fig. 2A-B (n=3-4); and Fig. 2A (phenformin; n=1)], but not on PRL, LH or FSH release (data not shown), mostly conserved between 4h and 24h. However, it should be noted that these inhibitory effects were apparently more pronounced after a short-term compared to a long-term incubation-period, being this inhibitory effect not statistically significant for baboon/macaque FSH-release (Fig. 2A-B) and macaque ACTH-release (Fig. 2B) after metformin treatment at 24h.

Direct effects of metformin on primate pituitary hormone expression.

Metformin treatment ($5x10^{-3}$M) clearly reduced the expression levels of *GH* and proopiomelanocortin (*POMC*, the ACTH precursor) at 24h, but not at 4h in baboon primary pituitary cells (Fig. 3). In contrast, it did not alter *FSHB* expression levels (Fig. 3), or those of other pituitary hormones (*PRL*, *LHB* or *TSHB*) at 4- or 24-h (Fig. 3).

Interaction of metformin with key regulators of GH, ACTH and FSH secretion: GHRH, ghrelin and GnRH in primate models

We next tested the direct effects of 4h of incubation with metformin alone or in combination with primary stimulatory factors of somatotrope, corticotrope or gonadotrope function, i.e. GHRH, ghrelin and GnRH; [22, 24, 29] (Fig. 4). This first revealed that, as expected, treatment with metformin alone inhibited GH, ACTH and FSH release, whereas GHRH, ghrelin or GnRH alone stimulated GH, ACTH and/or FSH release in primary pituitary cell cultures from baboons and macaques (Fig. 4). Interestingly, co-administration with

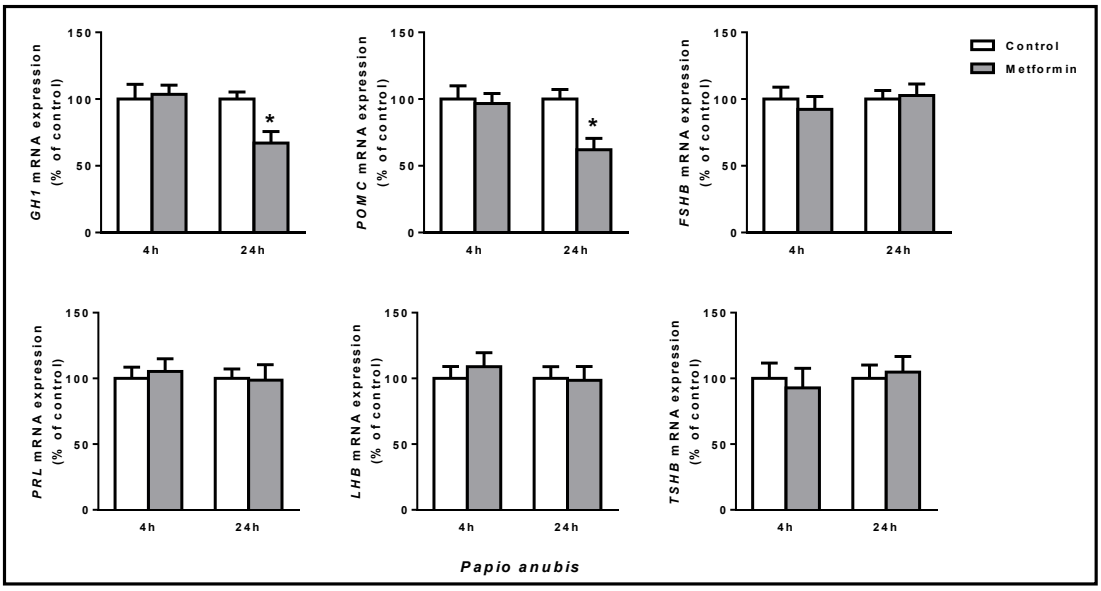


Fig. 3. Direct effect of metformin (5 mM) on mRNA expression of GH1, POMC, FSHB, PRL, LHB and TSHB in baboons. Data are expressed as percent of control (set at 100%) and represent the mean ± SEM (n=3 individual experiments, n=3-4 wells/experiments). Asterisks (*p<0.05) indicate values that significantly differ from their respective control values.

metformin did not impact the stimulatory actions of GHRH-stimulated GH release, ghrelin-stimulated GH or ACTH release, or GnRH-stimulated FSH release (Fig. 4).

Direct effects of metformin on primate pituitary cell viability

Treatment with metformin and phenformin did not alter cell viability in macaque primary pituitary cell cultures (Fig. 5). Similarly, pilot results from a single experiment performed in baboon primary pituitary cell cultures also indicated that metformin and phenformin treatment did not impact cell viability (data not shown). Moreover, as an indirect measurement of the maintenance of cell number after the treatments with metformin or phenformin in baboon and macaque primary pituitary cell cultures, we analyzed and observed that the recovery of total RNA in the vehicle-treated samples and in the biguanide-treated samples across experiments were markedly constant [RNA concentrations measure using the Ribogreen RNA quantification kit and also a NanoDrop Lite (Thermo Fisher Scientific, Wilmington, DE 19810, USA)], which indirectly confirmed that the treatment with these biguanides did not affect cell viability in normal primary pituitary cell cultures (data not shown).

Intracellular signaling pathways involved in the metformin-induced reductions in GH, ACTH and FSH release in the baboon model

The use of pharmacological inhibitors revealed that metformin inhibits GH, ACTH and FSH release through highly similar, if not identical signaling pathways (Fig. 6A-C, respectively). Specifically, our results indicate that the inhibitory effect of metformin on GH, ACTH and FSH release is likely mediated through mTOR, PI3K and intracellular Ca^{2+} influx, because incubation with specific blockers of these routes, but not with extracellular Ca^{2+}influx, AC or PLC inhibitors, completely blocked the inhibitory effect of metformin on GH, ACTH and FSH secretion (Fig. 6; metformin-columns). Interestingly, blockade of MAPK activity completely abolished the inhibitory effect of metformin on GH, but not ACTH or FSH, secretion (Fig. 6). Importantly, administration of these inhibitors alone did not modify basal GH, ACTH or FSH release (Fig. 6; control-columns). It should be noted that given the limited source of baboon cell preparations, we were able to study only some signaling routes, which were selected based on their importance on the functioning of multiple pituitary cell types [15]. Moreover, we were not able to study AMPK signaling using a similar approach since no specific and reliable pharmacological inhibitor is available to examine its function.

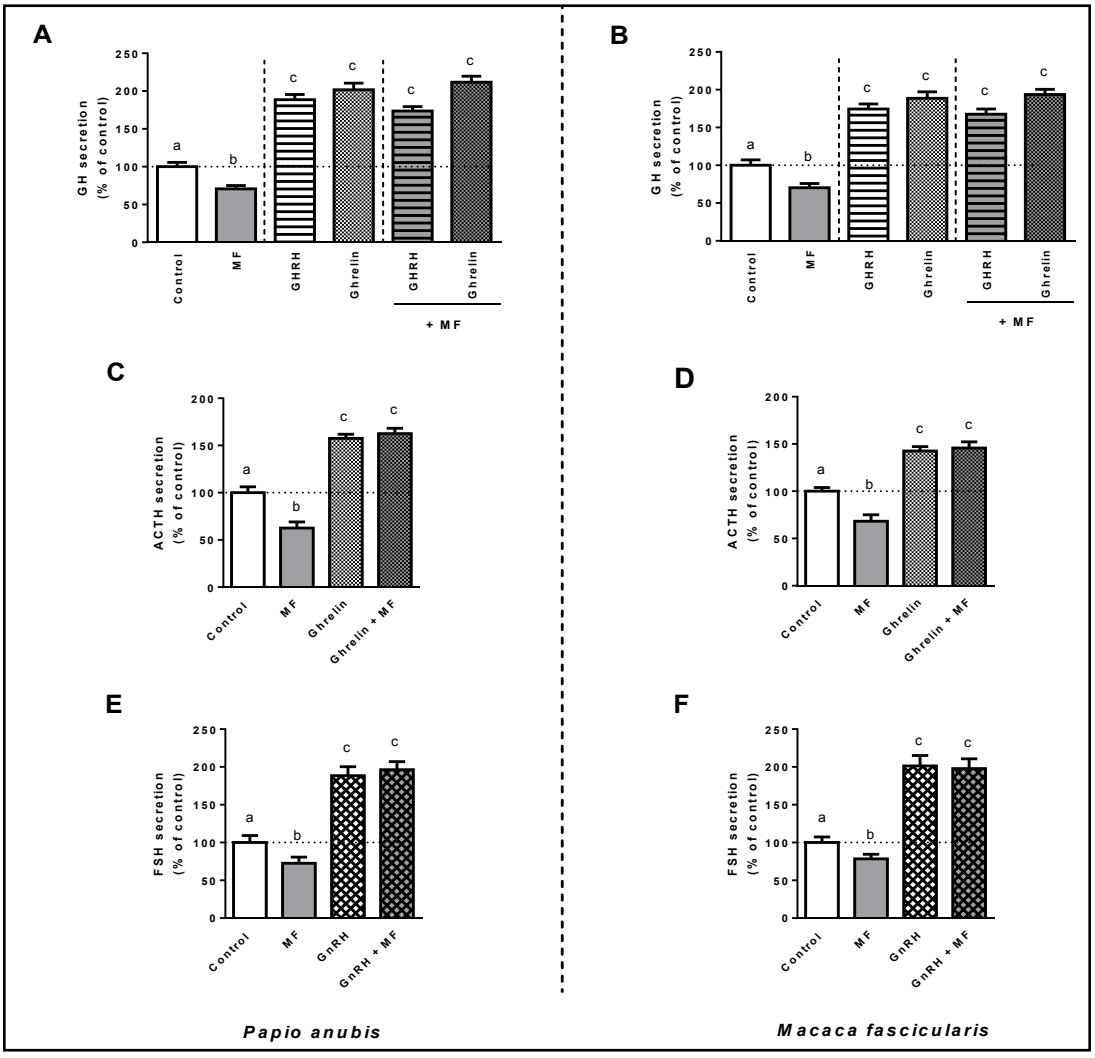
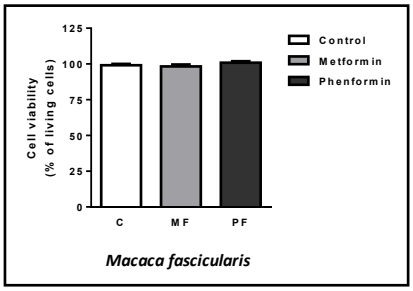


Fig. 4. Effect of 4h treatment of GHRH, ghrelin or GnRH (10 nM) in absence or presence of metformin (5 mM) on GH, ACTH and FSH secretion in primary pituitary cell cultures from baboons (A, C, E) and macaques (B, D, F). Data are expressed as percent of control (set at 100%) and represent the mean ± SEM (n=3-4 individual experiments, n=3-4 wells/experiments). Values that do not share a common letter (a, b and c) are statistically different.

Fig. 5. Effect of metformin (5 mM) on cell viability (24h) of primary pituitary cell cultures from macaques assessed by trypan-blue assay. Results are expressed as percent of control (set at 100%) and represent the mean ± SEM (n=3 individual experiments, n=3-4 wells/experiments).



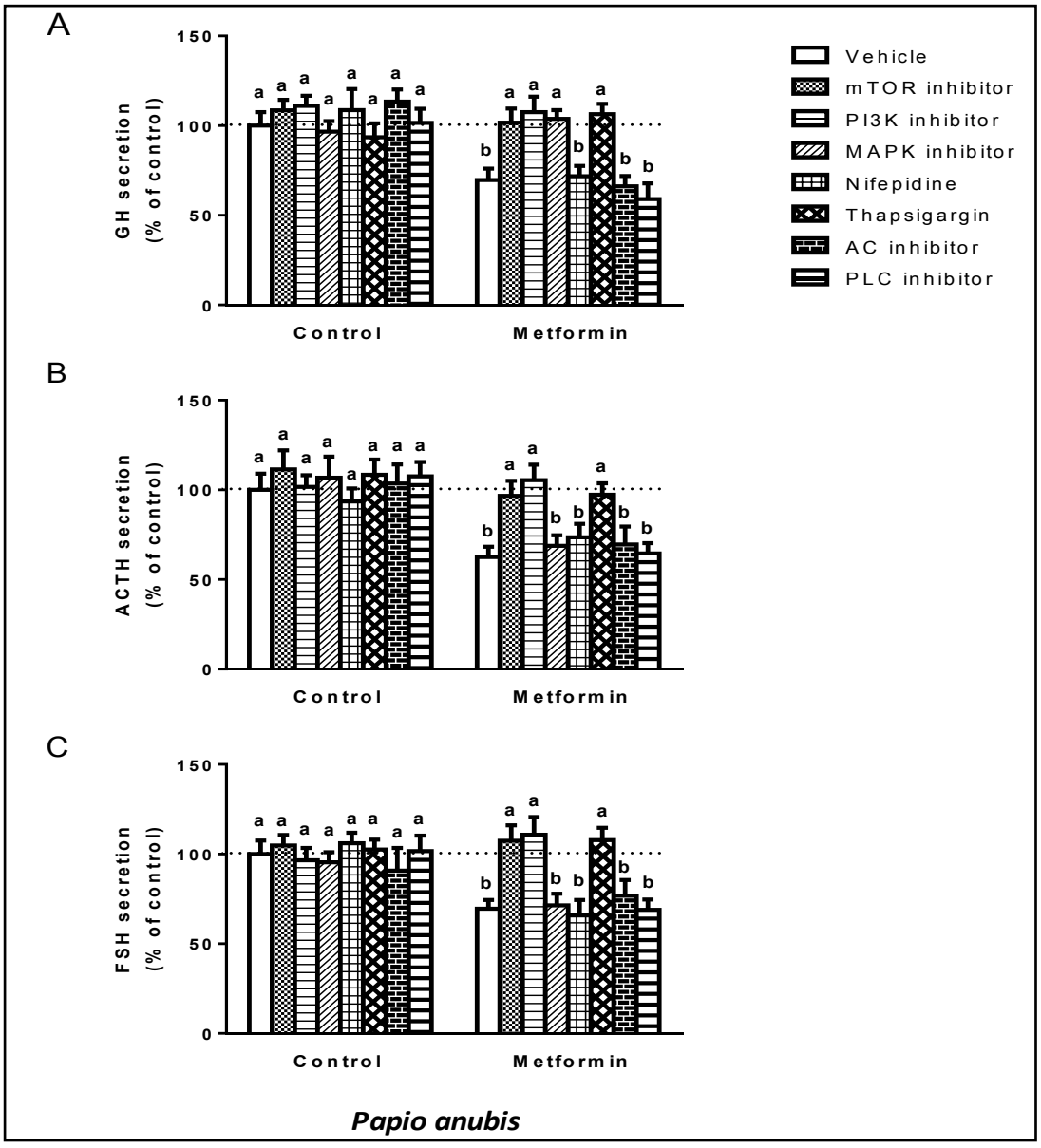


Fig. 6. Intracellular signaling pathways of metformin-regulated baboon GH (A), ACTH (B) and FSH (C). Effect of inhibition of mTOR (rapamycin; 1 μM), PI3K (wortmannin; 1μM), MAPK (PD-98,059; 10μM), extracellular Ca^{2+} channels (nifepidine; 1μM), intracellular Ca^{2+} channels (thapsigargin; 10μM), AC (MDL-12,330A; 10μM), and PLC (U73122; 50μM) on metformin-stimulated hormone release in primary pituitary cell cultures from baboons. Values are expressed as percent of vehicle-treated control without inhibitor (set at 100% within each experiment) and represent the mean ± SEM (n=3-4 individual experiments, n=3-4 wells/experiments). Values that do not share a common letter (a, b and c) are statistically different.

Direct effects of metformin on the expression of key receptors and a transcriptional factor involved in somatotrope, corticotrope and gonadotrope function.

Next, we studied the direct effect of metformin (24h of incubation) on the mRNA expression of a basic transcription factor for somatotropes, the pituitary transcription factor-1 (*POU1F1*) (Fig. 7A), and of selected key receptors controlling pituitary physiology (Fig. 7B). Metformin caused clear increases in the expression of key receptors associated to primary regulation of somatotrope, corticoptrope and gonadotrope function, including somatostatin receptor subtypes 2 and 5 (*SSTR2* and *SSTR5*; the two main pituitary receptor

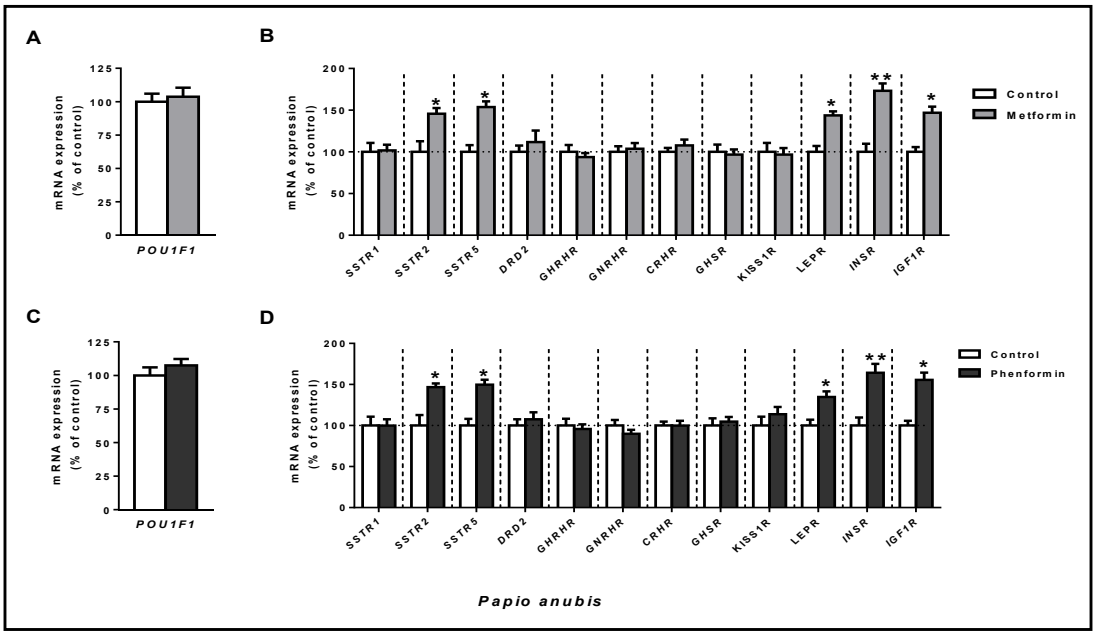


Fig. 7. Direct effect of metformin (n=3; 5 mM) and phenformin (n=1; 5 mM) on mRNA expression of (A, C) the pituitary transcription factor-1 (POU1F1); and (B, D) key receptors involved in the functioning of different pituitary cell-types (SSTR1, SSTR2, SSTR5, DRD2, GHRHR, GNRHR, CRHR, GHSR, KISS1R, LEPR, INSR and IGF1R) in primary pituitary cell cultures from baboons. Data are expressed as percent of control (set at 100%) and represent the mean ± SEM (n=3-4 wells/experiments). Asterisks (*p<0.05, **p<0.01) indicate values that significantly differ from their respective control values.

of the somatostatin system), and the receptors for leptin (*LEPR*), insulin (*INSR*) and IGF1 (*IGF1R*)] (Fig. 7B). Conversely, metformin did not alter the expression of *POU1F1* (Fig. 7A) or those of other receptors tested (somatostatin receptor subtype 1 (*SSTR1*), dopamine receptor subtype-2 (*DRD2*), GHRH-receptor (*GHRHR*), corticotropin-releasing hormone-receptor (*CRHR*), ghrelin-receptor (*GHSR*) or kisspeptin-receptor (*KISSR*). Moreover, results from a pilot, single experiment revealed that phenformin treatment exerted comparable effects to those observed with metformin on the expression of these receptor and *POU1F1* (Fig. 7C-D).

Discussion

Biguanides are synthetic drugs widely known by their antidiabetic properties, which seem to be primarily mediated by the inhibition of hepatic gluconeogenesis and the increase of glucose uptake in peripheral tissues, such as muscle or fat [2, 3]. However, biguanides are also being thoroughly studied due to their beneficial actions in the modulation of other critical (patho)physiological conditions such as the development of metabolic syndrome, cardiovascular disease or different types of cancer [4-10]. This pleiotropic nature of biguanides has also suggested the existence of additional sites of action (tissue targets) for these compounds. In this context, the "master endocrine gland", i.e. the pituitary gland, could also serve as a suitable target and mediator for the actions of biguanides, owing to its emerging role as a true sensor of alterations in whole body homeostasis and metabolism, by receiving, integrating and processing the information originating from central and peripheral signals, and appropriately conveying it to various key target endocrine and non-endocrine organs (i.e. liver, fat, muscle, etc) [15]. However, the information available about the effects that biguanides exert at the pituitary level is scarce, partially contradictory and somewhat controversial. Specifically, most of the data available mainly derives from early studies conducted in patients with different pathological conditions [i.e. polycystic ovary syndrome (PCOS), hypothyroidism, hyperprolactinaemia, or obesity], which have shown

that metformin could exert inhibitory actions on LH (but not FSH) TSH, PRL or GH levels, respectively [11, 12, 17, 18, 31-34]. But, our understanding of the effects of metformin, or other biguanides, in healthy humans remains a subject of intense debate since the available studies are quite limited and indicate that metformin does not seem to significantly impact plasma GH, LH or FSH levels [11, 12]; however, a caveat should be introduced at this point, because, although these early data undoubtedly have great value for the scientific community, most of this information was generated from studies using metformin for prolonged periods of time. Moreover, to the best of our knowledge, the direct, *in vitro*, effects of metformin at the pituitary level have been only reported in two studies showing that LH or FSH secretion levels were not altered in response to different doses of metformin after 48h of incubation in normal rat primary pituitary cells [26], whereas GH release was significantly reduced by metformin in tumoral GH3 cells and in cultured human GH-producing adenomas [35].

Thus, the question remains: can biguanides directly regulate pituitary function (basal and stimulated hormone release and hormone gene expression) under normal, non-pathological conditions in humans or non-human primates? To address this question, in the current study, we examined whether metformin and phenformin could directly impact hormone release/expression in primary pituitary cell cultures from normal baboons and macaques. These two primate species are of great interest for translational biomedical research, since they closely model human physiology, and have been frequently used to test a variety of hypotheses that cannot be directly tested in human subjects [19, 20]. Indeed, this approach provided here the first compelling evidence that metformin and phenformin inhibit somatotrope, corticotrope and gonadotrope function. Specifically, we showed that these biguanides act selectively to suppress basal GH, ACTH and FSH release and that this inhibitory action could be held over time. In fact, the maximal hormone release inhibition was already achieved (for GH, ACTH and FSH) after 4h of incubation, and although the inhibitory effect was still observed after long-term (24h) incubation for GH and ACTH, but not FSH, secretion, no further quantitative reduction was appreciable above the initial inhibition observed at 4h.

In addition, to our knowledge, this is the first report studying, in a cell culture system, the direct interaction between metformin with other primary regulators of somatotrope and corticotrope function. Specifically, we found that metformin treatment did not impact the stimulatory actions of GHRH-stimulated GH release, ghrelin-stimulated GH or ACTH release or GnRH-stimulated FSH release, which might suggest common mechanisms of action between those pituitary hormones-modulators and metformin (as will be further discussed below). It should be mentioned that this latter result is in contrast with the only previous report published to date indicating that GnRH-stimulated FSH and LH was reduced by metformin in rat primary pituitary cell cultures [26]. These discrepancies on the modulation of gonadotropes may be due, in part, to the age, sex and/or reproductive status of the donor, to the time of incubation (short vs. long periods), cell preparation and culture conditions, etc., but also, most likely, to fundamental differences in the physiology of gonadotropes from rat vs. primate species. Nevertheless, although the mechanisms and physiologic relevance behind the actions of metformin and phenformin observed in the present study remain unknown, our results are novel and demonstrate that one of the primary actions of these biguanides are confined to the regulation of basal, non-stimulated, GH, ACTH and FSH release. Moreover, these results demonstrate that these effects of both biguanides are conserved across the two primate models analyzed in this study, two species that closely model human genetics and physiology; thence, it is tempting to speculate that these biguanides may exert similar effects in anterior pituitary cells of humans, which set the stage for future investigations. In any case, our current data further extend previous observations, suggesting that the physiological actions of biguanides include a pituitary site of action as well.

Additionally, our data also indicated that the primary actions of metformin in the pituitary of baboons were not confined to the regulation of hormonal secretions, but also included regulation of the synthesis of different hormones. No previous studies have described the direct actions of metformin in the synthesis of all the anterior pituitary hormones in humans or non-human primates. Specifically, our data indicated that the observed inhibitory effects of metformin on baboon GH and ACTH secretion would be directly associated to and reinforced by a decrease in the expression of these hormones (i.e. *GH1* and *POMC*). Moreover, we also found that metformin did not alter *PRL*, *FSHB*, *LHB* and *TSHB* expression, which closely parallels the lack of effect observed at 24h of incubation in the release of these hormones.

Consequently, these data indicate that, whereas the pituitary actions of metformin extend to both hormonal synthesis and release in somatotrope and corticotropes, its effects on gonadotropes only seem to modulate secretory vesicle release, but not FSH expression. Importantly, our results indicate that the inhibitory actions of metformin and phenformin on pituitary cells cannot be attributed to an effect on cell viability or in the expression of the transcription factor Pit-1 since 24h-incubation with these biguanides did not alter cell viability or *POU1F1* expression in primate primary pituitary cell cultures.

Our report also provides the first analysis of the different intracellular signaling pathways that underlie the direct effects evoked by metformin on multiple anterior pituitary hormone secretions (i.e. GH, ACTH and FSH release). Specifically, the use of a standard pharmacological (inhibitory) approach revealed that the actions of metformin on GH, ACTH and FSH secretion are mediated by mTOR, PI3K and intracellular Ca^{2+} mobilization, but not by AC, PLC or extracellular Ca^{2+} influx. Of note, we also found that the inhibitory actions of metformin on GH, but not ACTH or FSH, secretion also require MAPK. At this point, it is important to mention that AMPK has been classically considered to be a central mediator of metformin effects in different tissues/organs 2; however, this contention remains controversial since numerous reports have also demonstrated AMPK-independent mechanism of actions for metformin [13, 14]. Indeed, although we were not able to determine the implication of AMPK using this pharmacological approach due to the lack of effective, and accepted, specific inhibitors of AMPK, our data further support the notion that metformin can act through AMPK-dependent and –independent pathways. Specifically, we found that three of the signaling pathways that are essential to mediated the actions of metformin at the pituitary (i.e. mTOR, PI3K and intracellular Ca^{2+} mobilization), have been previously described to be upstream or downstream of AMPK signaling pathway [36-40], which might suggests that metformin might act through these pathways in a AMPK-associated manner at the pituitary level. Additionally, we have also observed that metformin exerts inhibitory effects on GH secretion through pathways not linked with AMPK, such as MAPK signaling, which also demonstrate the existence of AMPK-independent mechanisms in somatotrope cells. Furthermore, the analysis of the different intracellular signaling pathways help to explain the reason why metformin treatment did not impact the stimulatory actions of ghrelin/GHRH /GnRH, as the actions of metformin are associated to the activation of similar and divergent signaling pathways to those evoked by these peptides. Specifically, ghrelin has been associated with the activation of multiple signaling cascades at the pituitary level in non-human primates, including PLC, protein kinase C (PKC), intracellular and extracellular Ca^{2+} and MAPK [15, 41]; while the signaling pathways associated to GHRH and GnRH include AC/cAMP/ protein kinase A (PKA), NOS/guanylate cyclase (GC) and intra-/extracellular Ca^{2+} [15]. Therefore, this report reveals that metformin, ghrelin, GHRH and GnRH exert their pituitary actions through distinct, but also common (i.e. intracellular Ca^{2+} and MAPK pathways), signaling pathways, which could explain, in part, why metformin treatment might not influence the stimulatory actions of these peptides at the pituitary level.

Our data indicated that the actions of metformin in the baboon pituitary also include regulation of the sensitivity of somatotropes, corticotropes and gonadotropes to some of their well-known regulatory factors (i.e. insulin, IGF1, leptin and somatostatin), some of which are tightly related with metabolic homeostasis [15, 29, 42-45]. In particular, metformin treatment provoked a significant increase in the expression of key receptors associated to primary inhibition of GH, ACTH and/or FSH secretion (e.g. *SSTR2*, *SSTR5*, *INSR* and *IGF1R*), which, in conjunction, might also be serving to enhance the inhibitory effects of metformin on the hormone expression and release of somatotropes, corticotropes and gonadotropes observed in these primate models.

Conclusion

Overall, the results of this report unveil the existence of various regulatory layers for metformin at the secretory, gene expression and signaling levels in the somatotrope, corticotrope and gonadotrope axes; however, there is a temporal dissociation between them. Specifically, signaling and secretory actions are rapid (4h) and can be sustained over time (24h), whereas gene expression effects (*GH1*, *POMC* and key regulatory receptors) necessarily require longer periods to be effective [24h, but not at 4h], and might represent an additional

regulatory mechanism to enhance the responsiveness of these pituitary cells to metformin. In this sense, the increase in the expression levels of insulin and IGF-1 receptors observed after 24h of incubation with metformin might represent a critical molecular, sensory element at the pituitary level associated to the anti-hyperglycemic and pro-metabolic character of biguanides, wherein metformin would increase the sensitivity to insulin and IGF-1 in different endocrine tissues, including the pituitary, to induce an increment in the uptake of glucose by the cells [46-49].

At the same time, the decrease in the secretion of GH and ACTH could be also one of the primary, beneficial, metabolic actions exerted by metformin to improve whole body homeostasis and metabolism since clinical and experimental studies have established that increased circulating levels of GH and glucocorticoids (secreted in response to pituitary ACTH) can lead to worsening of insulin resistance, glucose intolerance, overt diabetes mellitus [50]. In fact, given that GH is an important regulator of cellular and whole-body metabolism as well as body composition, and that elevation of circulating GH levels causes hyperinsulinemia and insulin resistance [50-55], a reduction in GH levels in response to metformin might be primary mechanism associated with the improved insulin sensitivity observed in response to metformin treatment. Therefore, overall, the results generated in the present study using two non-human primate models reinforce the contention that the pituitary is a primary site for the physiological actions of metformin, and that this gland would represent an additional, key target tissue and a true endocrine sensor contributing, in concert with other primary tissues (i.e. liver), to the well-known beneficial metabolic effects of biguanides in humans.

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Disclosure Statement

The authors have no competing interests.

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The pituitary gland is a major site of actions for statins: Potential antitumor effects of simvastatin in pituitary neuroendocrine tumor cells

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ABSTRACT

Pituitary neuroendocrine tumors (PitNETs) are the most abundant of all intracranial tumors (approx. 15%) and are associated with severe comorbidities. Transsphenoidal surgery is commonly the first-line therapy, but subsequent post-operative pharmacological therapy is often necessary to control disease symptoms. Unfortunately, a substantial proportion of patients are unresponsive or become resistant to available pharmacological therapies [somatostatin-analogues (SSAs)/dopamine-agonists], emphasizing the urgent need to identify new therapeutic options. Statins are well-known drugs commonly prescribed to treat hyperlipidemia/cardiovascular-diseases, but have also been related with other beneficial effects, including antitumor properties. The direct effects of statins on normal human pituitary or PitNETs are poorly know. Thus, here we aimed to explore the direct effects of statins, especially simvastatin, on key functional parameters (hormone-secretion/proliferation/cell-viability/signaling-pathways, etc.) in normal and tumoral primary pituitary cell-cultures from a primate model (*Papio anubis*) and different human PitNET-types [corticotropinomas/somatotropinomas/non-functioning pituitary-tumors (NFPTs)], respectively, and in rodent PitNET cell-lines (AtT20/GH3-cells). Additionally, combined effects of simvastatin with metformin (MF) or SSAs on AtT20/GH3 cell proliferation were also evaluated. All statins decreased proliferation in AtT20-cells, with simvastatin showing stronger effects. Indeed, simvastatin reduced cell-viability and/or hormone-secretion in all PitNETs-subtypes and cell-lines tested, and ACTH/GH/PRL/FSH/LH-secretion, but not TSH-secretion or ACTH/GH/PRL/FSH/LH/TSH-expression, in primate cell-cultures, likely through modulation of MAPK/PI3K/mTOR signaling pathways and expression of key receptors regulating corticotrope/somatotrope/lactotrope/gonadotrope function (i.e. down-regulation of GHRH-R/ghrelin-R/Kiss1-R). Addition of MF or SSAs did not enhance simvastatin antitumor effects. Interestingly, analysis of a second cohort of PitNETs patients revealed that pre-surgical statins treatment tended to be associated with less extrasellar-growth ($p=0.07$). Altogether, our data reveal a clear direct antitumor effect of simvastatin on PitNET-cells, thus paving the way to explore these compounds as a possible tool to treat PitNETs.

INTRODUCTION

Pituitary neuroendocrine tumors (PitNETs) represent approximately 15% of all brain tumors with a prevalence ranging from 1 in 865 to 1 in 2,688 people [1, 2]. Despite being often considered benign due to their extremely low metastatic capacity, PitNETs are accompanied by inadequate hormone secretion and mass effects, which cause severe comorbidities, from infertility and sexual dysfunctions, to growth abnormalities, hypogonadism, or hypopituitarism, and also increase mortality [3]. With the exception of prolactinomas, where dopamine agonists are the treatment of choice [4], transsphenoidal surgery is the first-line therapeutic option in patients harboring functioning PitNETs or non-functioning PitNETs presenting with compressive symptoms. In functioning tumors, surgical success is higher in patients with microadenomas (<10 mm), but in many cases there is an inadequate disease control, and subsequent pharmacological therapy and/or radiotherapy is necessary. To date, somatostatin analogues (SSAs) and dopamine agonists constitute the main pharmacological options to treat PitNETs patients [5]. Although these drugs have demonstrated great efficacy in reducing hormone hypersecretion and also inducing tumor shrinkage [5, 6], many studies have shown that some patients are (or become) unresponsive to these treatments [7, 8]. Accordingly, the search for new therapeutic options to enrich the pharmacological arsenal to treat PitNETs patients is urgently necessary.

Statins are well-established drugs, commonly prescribed in the clinical practice to treat hyperlipidemia and in cardiovascular or coronary heart diseases [9]. However, besides their cholesterol-lowering effects, statins have been related with an ample range of pleiotropic effects including immunomodulatory effects triggering the major histocompatibility complex, improvement of endothelial function and vasculoprotective effects, alteration of bone metabolism, etc. [9, 10], which confer to statins the ability to affect numerous tissue functions acting through cholesterol-dependent and -independent mechanisms [10]. Importantly, some studies have also related the use of statins with antitumor properties in different endocrine- and non-endocrine-related tumors [11-16]. Indeed, a meta-analysis analyzing several types of cancer

revealed that the use of statins seems to be beneficial for overall survival and cancer-specific survival [17]. For these reasons, there are several phase 1 and 2 clinical trials ongoing or finished using statins in different tumor pathologies including breast, prostate or lung cancer. Actually, although certain studies have related the use of statins with a potential risk of cancer [18], *in vitro* studies support the former, onco-protective contention, demonstrating that statins exert direct antitumor effects, including antiproliferative effects, inhibition of migration and invasion, pro-apoptotic actions and cancer-stem cells inhibition. These antitumor effects have been described in several tumor types including, among others, breast cancer, melanoma, and colon cancer [11, 12, 19]. Moreover, statins have been also related with an increase on apoptosis and a reduction on cell proliferation in pheochromocytoma and paraganglioma cells [14, 15], and results recently reported from our group have demonstrated a clear antitumor effect of statins in two neuroendocrine tumors-cell models (BON1 and QGP1 cells) [16]. However, to the best of our knowledge, the potential antitumoral actions of statins on PitNETs have not been reported to date. Likewise, although statins are known to modulate a wide variety of metabolic processes in humans, no studies have explored hitherto how statins can modulate directly the function of the normal anterior pituitary cell types in humans or in a close, primate species model.

Based on the above, this study was devised to explore the direct effects of statins, specially simvastatin, on key functional parameters (cell viability/proliferation, hormone secretion, and intracellular signaling pathways) in primary cell cultures from different human PitNET subtypes, including corticotropinomas (ACTHomas), somatotropinomas (GHomas) and non-functioning pituitary tumors (NFPTs), as well as in two rodent pituitary cell line models (AtT-20 and GH3). In addition, we also analyzed the ability of simvastatin to influence relevant functional parameters (hormone secretion, cell viability, and modulation of expression of key genes and intracellular signaling pathways) in primary cell cultures from normal pituitary of baboons (*Papio anubis*), a species that closely resemble human physiology [20, 21]. Moreover,

we aimed to assess the putative association between pre-surgical statin treatment and clinical and tumor parameters in an ample cohort of PitNET patients.

MATERIALS AND METHODS

Drugs and reagents

All reagents, inhibitors of intracellular signaling pathways and drugs, including all statin types, used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. Octreotide was obtained from GP-Pharm (Barcelona, Spain) and pasireotide was generously provided by Novartis (Barcelona, Spain). Metformin (MF) was used at 10 mM and SSAs at 100 nM. All doses were selected based on previous studies [16, 22-24].

Patients, tumor samples and primary cell cultures

This study was carried out within a project approved by our Hospital Research Ethics Committees, was conducted in accordance with ethical standards of the Helsinki Declaration of the World Medical Association, and written informed consent was obtained from each patient.

Human PitNETs samples for *in vitro* (primary cell cultures) studies were collected during transsphenoidal surgery from 19 patients (7 ACTHomas [mean age: 46 (21-79); 86% women], 8 GHomas [mean age: 49 (36-66); 63% women] and 4 NFPTs [mean age: 58 (41-71); 75% women]. Each pituitary sample subtype was evaluated by an expert anatomo-pathologist and confirmed by immunohistochemistry. Additionally a molecular confirmation screening of relevant genes using quantitative real-time PCR (qPCR) was performed, as previously described [24-27]. In all cases, samples were placed in sterile cold medium (supplemented S-MEM, Gibco, Madrid, Spain) and dispersed into single cells following the methods and reagents previously described [24, 26]. Cells (10.000-150.000 cells/well) were plated onto 96-, 24- or 12-well plates and incubated with serum-free medium alone (controls) or serum-free media containing simvastatin (1nM-10μM) using the material and reagents previously described [24, 26].

Additionally, clinical data from a second cohort of PitNET patients (n=132; 42 ACTHomas, 28 GHomas and 62 NFPTs) were collected to explore the putative association between treatment with statins and clinical outcomes of patients harboring different PitNETs subtypes (Table 1).

Pituitary tumor cell lines

Two commonly used rodent pituitary tumor cell lines, the mouse pituitary corticotrope-derived cell line AtT-20/D16v-F2 (ATCC® CRL-1795™) and the rat pituitary somatotrope-derived cell line GH3 (ATCC® CCL-82.1™), were used. Both cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) complemented with 10% FBS, 100 U/ml penicillin/streptomycin, 0.024 M of HEPES, and maintained at 37ºC and 5% CO2, under sterile conditions. Both cell lines were checked for mycoplasma contamination by PCR [28].

Animals, pituitary collection and primary cell cultures

Primate (Olive Baboon, *Papio anubis*; n=4, 8-10 years of age) pituitaries were obtained from females within 15 min after sodium pentobarbital overdose as previously reported [29]. These baboons represent control animals from a breeding colony, all under Institutional Animal Care and Use Committee approved studies conducted by other University of Illinois at Chicago investigators. All experimental protocols were approved by University of Illinois at Chicago institutional committees. Right after the animals were euthanized, pituitaries were excised, placed in sterile cold (4 °C) basic media (α-MEM with 0.15% BSA, 6-nM HEPES, 10-IU/mL penicillin, and 10-µg/mL streptomycin) and immediately transported to the laboratory in sterile conditions. Then, distal pituitary was isolated, washed twice in fresh media, cut into small pieces with surgical blades and dispersed into single cells, as previously reported [30]. Cells (25.000-100.000 cells/ well) were plated onto 48- or 24-well plates and incubated with serum-free medium alone (controls) or serum-free media containing simvastatin (1nM-10µM) using the material and reagents previously described [22].

To avoid fibroblast contamination, suspensions of dispersed human PitNETs cells or baboon pituitary cells were filtered and cultured in media with D-Valine (replaced for L-valine) to selectively inhibit fibroblast proliferation/overgrowth. In addition, visual inspection of primary cell cultures at the time of experimental assays showed no sign of cells displaying the typical fibroblast-like morphology.

Analysis of cell proliferation/viability

Cell viability of PitNETs cell cultures (10,000 cells/well in 96-well plates: 3 wells/treatment) was evaluated every 24h until 72h in response to different statins using Alamar-blue reagent (Invitrogen, Madrid, Spain). Similarly, proliferation rate was evaluated in GH3 or AtT-20 cell lines (6,000 cells/well were seeded in 96-well plates) in response to simvastatin, MF, SSAs (octreotide and pasireotide) or in response to the combination of simvastatin with MF or SSAs. Treatments were daily refreshed after each measure. Reduction of Alamar-blue was quantified using a FlexStation III system (Molecular Devices, Sunnyvale, CA), as previously reported [25, 27, 31]. Moreover, in order to determine if simvastatin altered cell viability in baboon primary pituitary cell cultures (25,000 cells/well in 48-well plates: 3 wells/treatment), trypan blue assay (Sigma, St. Louis, MO) was used as previously reported [22], following the manufacturer's instructions.

Measurement of hormone release

Human primary PitNET cell cultures (150,000 cells/well) or baboon primary pituitary cell cultures (100,000 cells/well) were incubated with media alone (controls) or simvastatin (10μM) for 24h. After this time, media were collected and hormone secretion was measured using human commercial ELISAs [ACTH, GH, PRL, FSH, LH, TSH or Chromogranin A; reference numbers: EIA-3647; EIA-1787, EIA-1291, EIA-1288, EIA-1289, EIA-1790 and EIA-4937, respectively (DRG, Mountainside, NJ)]. All the assays were performed following the manufacturer's instructions where the information regarding specificity, detectability and reproducibility for each of the assays can be accessed at the websites of the indicated company.

RNA isolation, reverse-transcription and qrtPCR of baboon transcripts

Primary pituitary cell cultures from baboons were processed for recovery of total RNA, subsequent quantification of RNA amount, reverse-transcription (RT) and application of a quantitative real-time PCR using kits, primers and methods previously described [30]. Samples

were run against synthetic standards (1-10^6 copies) for each transcript of interest [somatostatin-receptor subtype (SST_1, SST_2, SST_3 and SST_5), GH-releasing hormone receptor (GHRHR), ghrelin-receptor (GHSR), corticotropin-releasing hormone receptor (CRHR), gonadotropin-releasing hormone receptor (GNRHR) and kisspeptin-receptor (KISS1R)] to estimate mRNA copy number, and a No-RT sample was used as a negative control. As previously reported [22, 30, 32], to control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, mRNA copy numbers of the baboon transcripts analyzed were adjusted by PPIA (cyclophilin-A; used as housekeeping gene) expression, where baboon cyclophilin-A mRNA levels did not significantly vary between experimental groups (data not shown).

Measurement of signaling pathways

Due to the limited number of primary PitNET cells obtained after dispersions, AtT-20 and GH3 cell lines were used to further explore the signaling pathways involved in the response to simvastatin in pituitary tumor cells. Thus, 500,000 cells/well were plated in 12-well plates and incubated 10 min, 4h and 24h with simvastatin or vehicle-treated controls. Proteins were extracted, separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Darmstadt, Germany), as previously reported [27]. Then, blocked membranes were incubated with different primary antibodies [phospho-ERK1/2 (Ref. CS4370), ERK1/2 (Ref. SC-154), phospho-Akt (Ref. CS9271S) and Akt (Ref. CS9272)] and the appropriate secondary antibody (anti-rabbit antibody from Cell Signaling, Danvers, MA, USA). Proteins were developed using an enhanced chemiluminescence detection system (GE Healthcare, Madrid, Spain) with dyed molecular weight markers. A densitometry analysis of the bands was carried out with ImageJ software. Relative phosphorylation was estimated from normalization of p-ERK1/2 or p-Akt against the total ERK1/2 or Akt, respectively. It should be mentioned that, as previously observed, p-Akt could not be detected in GH3 cells using this antibody.

Additionally, in order to study the intracellular signaling pathways involved in the simvastatin-mediated pituitary actions in baboon cells, we used inhibitors of different

intracellular signaling pathways [mTOR (rapamycin; 10 nM) and PI3K (wortmannin; 1 μM), MAPK (PD-98,059; 10 μM), extracellular Ca^{2+} L-type channels (nifedipine; 1 μM) and intracellular Ca^{2+} channels (thapsigargin; 10 μM); 100,000 cells/well in 24-well plates: 3 wells/treatment; 24h incubation] using the material, reagents and the doses of inhibitors previously described [22, 30, 32].

Statistical analysis

Samples from all groups within an experiment were processed at the same time. All data are expressed as mean ± SEM. Statistical differences were assessed by paired parametric t-test or one-way ANOVA [or two way ANOVA when the intracellular signaling pathways, with treatments with and without (controls) specific inhibitors, were studied] followed by Dunnett´s test for multiple comparison (according to normality evaluated by Kolmogorov-Smirnov test). The values obtained were compared with vehicle-treated controls (set at 100%) to normalize values within each treatment and minimize intragroup variations in the *in vitro* experiments (i.e. different age of the tissue donor, stage of the estrus cycle and/or to the metabolic environment, etc.). All experiments were done in a minimum of three independent primary cultures from different patients, baboons or three independent passages of cell lines (3-4 replicates/treatment per experiment), unless otherwise indicated. P-values ≤ 0.05 were considered statistically significant. A trend for significance was indicated when P values ranged between >0.05 and <0.1. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software; La Jolla, CA, USA) or GB-STAT software package (Dynamic Microsystems, Silver Spring, MD).

RESULTS

Direct effects of statins on proliferation, cell viability and hormone secretion in PitNETs cells and cell lines

Concentration-response experiments carried out with different statins (simvastatin, atorvastatin, lovastatin, or rosuvastatin) in corticotrope AtT-20 cells demonstrated statin- and dose-dependent effects on cell proliferation. Specifically, nM doses (1nM or 100nM) of different statins (simvastatin, atorvastatin, lovastatin and rosuvastatin) did not alter proliferation in AtT-20 cells at any of the incubation times tested (24-72h; Figure-1). In contrast, 10μM of simvastatin, lovastatin and rosuvastatin clearly reduced cell proliferation at 24, 48, and 72h of incubation, while the same dose of atorvastatin reduced proliferation only at 72h. In general, simvastatin was the most effective statin reducing cell-proliferation at 72h (64.5% of reduction compared to 28.6%, 40%, and 40.5% for atorvastatin, lovastatin, and rosuvastatin, respectively; Figure-1). These results, coupled to the fact that simvastatin was the statin most related to antitumor properties in pre-clinical and clinical studies [11, 33-35], prompted us to select the 10μM dose of simvastatin for further experiments.

We next tested the antitumor effects of simvastatin (10μM) in primary cell cultures from different PitNETs subtypes. Remarkably, simvastatin clearly reduced cell viability at 24, 48 and 72h of incubation in ACTHomas and NFPTs, and also at 72h in GHomas (Figure-2A). Similarly, simvastatin (24h) also decreased ACTH and GH secretion in ACTHomas and GHomas, although this reduction only reached statistical significance in ACTHomas (n=4 and 2; 18.6 % and 20.5 % of reduction, respectively; Figure-2B). Moreover, simvastatin decreased chromogranin-A secretion by 50 % in NFPTs cell culture (Figure-2B).

Direct effects of simvastatin on primate pituitary hormone expression/release and cell viability

Increasing doses of simvastatin (1 nM-10 μM) for 24h in cultured baboon pituitary cells decreased the spontaneous secretion of ACTH, GH, PRL, FSH and LH, but not TSH, in a dose-dependent manner (i.e. at 10 μM in all cases, and also at 1μM in the case of GH and PRL release; Figure-3A). Therefore, the dose of simvastatin that caused a maximal decrease of ACTH, GH, PRL, FSH and LH release, 10 μM, was chosen to further analyze the action of the peptide on primate pituitary function. Interestingly, treatment with simvastatin did not alter the gene expression of any of the primate pituitary hormones [pro-opiomelanocortin (POMC, the precursor of ACTH), GH, PRL, FSH, LH or TSH; (Figure-3B)] and did not influence cell viability of cultured primate primary pituitary cells (Figure-3C).

Effect of simvastatin on intracellular signaling pathways in tumor and normal pituitary cells

Due to the limited number of cells obtained after cellular dispersion of PitNET tissues, we initially used AtT-20 and GH3 cells to assess the signaling pathways modulated by simvastatin pituitary tumor cells. First, we confirmed that simvastatin (10 μM) significantly reduced cell proliferation at 24, 48, and 72h in corticotrope AtT-20 cells (Figure-4A, left-panel; which includes a higher number of experiments than those presented in Figure-1) and in somatotrope GH3 cells (Figure-4A, right-panel), being these effects comparable in both pituitary cell lines (Figure-4). Then, we measured selected signaling pathways closely associated with proliferation in tumor pathologies, including PitNETs [36-38]. Our results show that simvastatin significantly decreased the phosphorylation levels of ERK1/2 in AtT-20 and GH3 after short-time (4h) and long-time (24h) incubation periods, respectively. In contrast, simvastatin did not alter phosphorylation levels of Akt in AtT-20 cells (Figure 4B).

As shown in table 2, simvastatin was also able to modulate the dynamics of free cytosolic calcium concentration ($[Ca^{2+}]_i$) in single cells derived from PitNETs. Specifically, simvastatin modestly suppressed $Ca^{2+}{}_i$ levels in a small number of cells in ACTHomas (11%),

and NFPTs (9%) cells (evoking a reduction in $[Ca^{2+}]_i$ of 25% and 20%, respectively), with no effect in GHomas.

To better understand the effects observed in response to simvastatin in normal primary pituitary cell cultures from primates, we explored the underlying signaling pathways using pharmacological inhibitors (Figure-5A). However, given the limited source of baboon cell preparations, we were able to study only some key selected signaling routes. This approach revealed that blockade of PI3K and mTOR activity completely abolished the inhibitory effect of simvastatin on ACTH, GH, PRL, FSH and LH release (Figure-5A). Interestingly, our results also indicate that the inhibitory effect of simvastatin on ACTH, GH and PRL release, but not FSH and LH secretion, is also mediated through MAPK in normal primary pituitary cell cultures (Figure-5A). Finally, we could also test, in two baboon cell cultures experiments, the role played by Ca^{2+} signaling in simvastatin-mediated actions of pituitary hormone secretion. These results revealed that the inhibitory effects of simvastatin on ACTH, GH, PRL, FSH and LH release were not mediated through extra-/intra-cellular Ca^{2+} influx because incubation with specific blockers of these routes did not alter the inhibitory effect of simvastatin on the secretion of these hormones (data not shown), consistent with the minor effect of simvastatin on Ca^{2+}signaling in PitNETs cells (Table-2).

Effects of simvastatin on the expression of key receptors in corticotrope, somatotrope, lactotrope and gonadotrope function

Next, we studied the direct effect of simvastatin (24h of incubation) on the mRNA expression of selected receptors controlling pituitary physiology (Figure-5B). Simvastatin significantly reduced the expression of key receptors associated to primary regulation of corticoptrope, somatotrope, lactotrope and gonadotrope function, including GHRH-receptor (GHRHR), ghrelin-receptor (GHSR) and kisspeptin-receptor (KISSR). Conversely, simvastatin did not alter the expression of the receptors of the somatostatin system, SST_1, SST_2, SST_3 and SST_5, or those for CRH (CRHR) and GnRH (GNRHR) (Figure-5B).

Combined effect of simvastatin with metformin (MF) or SSAs in pituitary cell lines.

We also employed AtT-20 and GH3 cells to evaluate the potential antiproliferative effects of the combined administration of simvastatin with MF, an antidiabetic drug commonly used to treat type-2 diabetes mellitus, which we recently demonstrated to exert antitumor actions in PitNETs [38], as well as with first-generation SSAs (octreotide and pasireotide). This revealed that MF clearly reduced cell proliferation after 24, 48, and 72h of incubation in GH3 cells (Figure-6A, right-panel). Whereas, in AtT-20 cells, in spite of the numerical reduction observed, the inhibitory action of MF did not reach statistical significance (Figure-6A, left-panel). In line with this, the combination therapy of simvastatin and MF did not modify the inhibitory actions of simvastatin tested alone in AtT-20 cells (Figure-6A, right-panel). In contrast, the effect of this combination therapy was markedly higher in GH3 cells after short-term incubation (24h) compared with simvastatin or MF in monotherapy, but this effect was not observed after longer incubation periods (48 or 72h; Figure-6A, left-panel). Furthermore, the combination of simvastatin with octreotide or pasireotide did not significantly impact the reduction elicited by simvastatin in AtT-20 and GH3 cells (figure-6B).

Clinical relevance of treatment with statins in patients with PitNETs.

To assess the clinical context of the potential interplay between statins treatment and PitNETs, we explored the putative association between pre-surgery treatment with statins and clinical outcomes of patients harboring different types of PitNETs. Clinical characteristics were compared between patients with PitNETs treated or not with statins (Table 1). These comparisons revealed that patients with NFPTs and treated with statins (n=12) showed a trend to have less extrasellar growth compared with patients not treated (n=36, p=0.07). Conversely, none of the other clinical parameters evaluated (i.e. BMI, lesion type, tumor size, symptoms including cephalea or visual alterations, use of other treatments, disease persistence after

surgery, etc.) revealed significant associations with statins treatment in this pilot, exploratory patient cohort.

DISCUSSION

Evidence gathered over the last years has prompted an emerging interest in exploring the putative role of statins in the development and progression of different endocrine- and non-endocrine-related tumors [11-16], particularly in view of recent meta-analysis analyzing several types of cancer, which revealed that the use of statins improve overall survival and cancer-specific survival [17]. However, to date, the possible antitumoral actions of these compounds on PitNETs, as well as the direct effects of statins in normal pituitary cells, had not been reported. In this study, we demonstrate for the first time that statins, especially simvastatin, exerts relevant antitumor actions (i.e. reduction in cell viability/proliferation and hormone secretion) in cell cultures from different human PitNET types (ACTHomas, GHomas and NFPTs) and representative pituitary cell lines (ACTH- and GH-producing cells) mainly through the modulation of MAPK (i.e. ERK1/2) pathway. Moreover, we found that simvastatin also decreases pituitary hormone secretion, but not gene expression, or cell viability, in primary normal pituitary cell cultures from baboons through the modulation of PI3K, mTOR and MAPK. Therefore, these results suggest that the medical use of simvastatin could be beneficial to improve key clinical/pathological aggressiveness parameters in patients with PitNETs, in that this drug might reduce cell viability/proliferation of ACTHomas, GHomas and NFPTs, but not normal pituitary cells, as well as decrease hormone secretion in normal and tumor pituitary cells.

Our first set of studies revealed that different statins, specially simvastatin, can reduce cell proliferation in AtT-20 and GH3 cell lines, as well as cell viability in human PitNET cells, while they did not alter survival of normal pituitary cells from primates. In line with this, an antiproliferative capacity of statins has been described in different tumor pathologies, including neuroendocrine tumor cells [14, 16, 34, 35]. In our study, all statins tested were able to significantly decrease proliferation in AtT20 cells, with simvastatin showing slightly stronger effects. These results are in agreement with the reduction of cell proliferation previously observed in murine pheochromocytoma cell lines [14], and with a recent study from our group

in human pancreatic NET cell lines [16], wherein simvastatin was the most efficient statin in decreasing this parameter. Interestingly, compared with GHomas, the effects of simvastatin were more evident in ACTHomas and NFPTs, which are the most resistant tumor types to the currently available therapeutic options [24, 39]. Thus, our results unveil a previously unknown antitumoral potential of statins, particularly simvastatin, in PitNETs.

Furthermore, exploration of the effect of simvastatin on hormone secretion in different PitNETs subtypes and in normal pituitary cells demonstrated that simvastatin can reduce ACTH, GH and chromogranin-A secretion in ACTHomas, GHomas and NFPTs, respectively, as well as ACTH, GH, PRL, FSH and LH secretion in normal pituitary cells from primates. These results are also of potential clinical relevance, in that hypersecretion of pituitary hormones causes serious comorbidities in patients with PitNETs [3]. Hence, a reduction of hormone secretion from tumor cells in response to simvastatin would be clearly beneficial for patients harboring these pathologies. On the other hand, the nearly general inhibitory action of statins on pituitary hormone secretion from normal primary cells from a primate species closely resembling human physiology is intriguing, and it unveils an unpredicted potential endocrine action of long-term statin use in patients *in vivo* that certainly deserves further examination in the future.

Mechanistic assays provided further insights into the signaling pathways underlying the effects of simvastatin in normal pituitary and PitNET cells. Specifically, functional assays showed that the inhibitory action of simvastatin in ACTHoma and NFPT cells may not be mainly mediated through $Ca^{2+}{}_i$ signaling, in that only ~10% of the cells displayed changes in their $[Ca^{2+}]_i$ in response to simvastatin, and also, that this pathway would not be involved in the actions of simvastatin in GHomas. On the contrary, our results strongly indicate that the antitumoral actions of simvastatin in ACTHomas and GHomas are mediated through the modulation of the MAPK signaling pathway, according to the reduction on the phosphorylation levels of ERK1/2 observed in AtT-20 and GH3 cells in response to simvastatin. The relevance of this signaling pathway on simvastatin-mediated effects in PitNET cells compares nicely with

previous studies in different tumor pathologies, where the MAPK pathway is also essential for the actions of statins [11, 14, 16]. Interestingly, a decrease in the phosphorylation levels of this pathway has been commonly associated to a reduction of cell proliferation in different PitNET subtypes [37].

In the same line, the present report also provides the first analysis of the different intracellular signaling pathways that underlie the direct inhibitory effects evoked by simvastatin on the secretion of multiple anterior pituitary hormones from a primate model (i.e. ACTH, GH, PRL, FSH and LH). Specifically, the use of a standard pharmacological (inhibitory) approach revealed that the actions of simvastatin on ACTH, GH, PRL, FSH and LH secretion are mediated by mTOR and PI3K, but do not appear to involve intracellular Ca^{2+} mobilization or extracellular Ca^{2+} influx. Interestingly, we also found that the inhibitory actions of simvastatin on ACTH, GH or PRL secretion also require MAPK, which is in accordance with the results previously observed with the modulation of ERK1/2 in ACTHoma and GHomas cells. In addition, our data revealed that simvastatin actions in primate pituitary would also include regulation of the sensitivity of pituitary cells to some of their well-known regulatory factors (i.e. GHRH, ghrelin and kisspeptin). Thus, simvastatin treatment evoked a significant downregulation in the expression of key receptors associated to the stimulation of ACTH, GH, PRL, FSH and LH secretion (e.g. GHRHR, GHSR and KISS1R) [29, 30, 40, 41], which, in conjunction, might also be serving to enhance the inhibitory effects of simvastatin on hormone release from corticotropes, somatotropes, lactotropes and gonadotropes observed herein.

Finally, taking into account that: 1) our group has recently reported the antitumor actions of MF in cells from NETs and different PitNETs subtypes [38]; 2) there are numerous ongoing or finished clinical trials with simvastatin as monotherapy or in combination with other agents [42]; and, 3) simvastatin and MF are commonly used in clinical practice, and both drugs have demonstrated antitumor properties, we sought to ascertain the potential antitumor effects of the combination therapy of simvastatin and MF in PitNET cell lines. Our results revealed that, in general, the addition of MF did not modify the effects of simvastatin in different pituitary tumor

cells (with the exception of an additive effect at 24h in GH3 cells that disappeared at 48-72h). Along the same lines, SSAs are also frequently used alone or in combination with other pharmacological options (dopamine agonists or pegvisomant) to control PitNETs symptoms [43, 44]. In our study, the combination of simvastatin and SSAs did not result in any additive effect in AtT-20 or GH3 cells. All these evidences might suggest that simvastatin, MF, and SSAs are sharing mechanisms of action in pituitary tumor cells. Indeed, several reports have linked the statins with the activation of AMP-activated protein kinase (AMPK; considered the central mediator of MF actions) in numerous cancer types [45-47]. Likewise, the antitumor effects of simvastatin observed in this and other studies have been associated to the dysregulation of several signaling pathways including Ras-MAPK, PI3K-Akt, and AMPK/Akt/mTOR pathways, all of them also involved in the effects of SSAs and MF [22, 37, 38, 48].

Finally, a pilot, exploratory analysis of the potential clinical context of statins use in PitNET patients did not reveal major associations between use of statins and clinical variables in our cohort of patients, except for a non-significant trend to lower extrasellar growth in NFPTs. However, the limited number of patients treated with statins and the retrospective observational nature of the analysis likely limit these results, and this issue certainly warrants further investigation.

In conclusion, our study provides primary evidence that statins, especially simvastatin, exert effective antiproliferative and antisecretory effects in different PitNET subtypes, without altering the viability of normal pituitary cells. Furthermore, we present mechanistic insights into the plausible signaling pathways underlying the inhibitory actions of simvastatin on cell survival and hormone release, which may partially overlap with those employed by MF or SSAs, as their combination with simvastatin did not elicit relevant additional antitumor effects. Therefore, our data unveil clear antitumoral direct effects of simvastatin on PitNET cells, thereby opening the possibility to explore these compounds as a novel tool to enrich the limited pharmacological arsenal available to treat patients harboring PitNETs.

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FIGURE CAPTIONS.

Figure 1: Dose-response experiment of cell proliferation in response to different statins (simvastatin, atorvastatin, lovastatin and rosuvastatin) at 1 nM, 100 nM and 10 μM in AtT20 cells (n=4), measured by Alamar-blue reduction. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* $p<0.05$; ** $p<0.01$) indicate statistically significant differences.

Figure 2: Functional assays in response to simvastatin in PitNETs primary cell cultures. (A) Effect of simvastatin (10 μM) on cell viability (24-72h) in primary ACTHomas (n=5), GHomas (n=6), and NFPTs (n=4), measured by Alamar-blue reduction. (B) Effect of simvastatin (24h treatment) on ACTH, GH, and chromogranin A secretion in ACTHomas (n=5), GHomas (n=2) and NFPTs (n=1), respectively, determined by commercial ELISA kit. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) indicate statistically significant differences. In cases where less than three experiments were performed, no significance tests were performed.

Figure 3: Functional assays in response to simvastatin in normal pituitary cells. (A) Dose-response (24h) of simvastatin on the secretion of ACTH, GH, PRL, FSH, LH and TSH in primary pituitary cell cultures from baboons (n=4). (B) Effect of simvastatin (10 μM) on mRNA expression of POMC, GH, PRL, FSH, LH and TSH (n=3). (C) Effect of simvastatin (10 μM) on cell viability (24h) of primary pituitary cell cultures from baboons assessed by trypan-blue assay (n=4). Data are expressed as percent of control (set at 100%) and represent the mean ± SEM. Values that do not share a common letter (a, b and c) are statistically different.

Figure 4: Measurement of cell proliferation and signaling pathways in pituitary cell lines. (A) Effect of simvastatin (10 μM) on cell proliferation (24-72h) in corticotropinoma AtT-20 (n=6) and somatotropinoma GH3 (n=8) cell lines, measured by Alamar-blue reduction. (B) Representative Western Blots and quantification of levels of pERK1/2/ total ERK1/2 and p-Akt/

total Akt in response to simvastatin in AtT-20 and GH3 cells (10 μM; n=3). Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) indicate statistically significant differences.

Figure 5: Direct effect of simvastatin (10 μM) on intracellular signaling pathways and mRNA expression levels of key genes. (A) Effect of inhibition of mTOR (rapamycin; 1 μM), PI3K (wortmannin; 1μM) and MAPK (PD-98,059; 10μM) on simvastatin-stimulated hormone release in primary pituitary cell cultures from baboons (n=3). (B) Effect of simvastatin on mRNA expression of key receptors involved in the functioning of different pituitary cell types in primary pituitary cell cultures from baboons (n=3). Values are expressed as percent of vehicle-treated control without inhibitor (set at 100% within each experiment) and represent the mean ± SEM. Values that do not share a common letter (a, b and c) are statistically different. Asterisks (*$p<0.05$, **$p<0.01$) indicate values that significantly differ from their respective control values.

Figure 6: Measurement of cell proliferation/viability (24-72 h) in response to simvastatin alone or in combination with metformin (10 mM) or SSAs (octreotide and pasireotide; 100 nM) in pituitary cell lines, measured by Alamar-blue reduction. (A) Effect of simvastatin alone or in combination with metformin in AtT-20 (n=8) and GH3 (n= 4) cells. (B) Effect of simvastatin alone or in combination with SSAs in AtT-20 and GH3 (n=4) cells. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) indicate statistically significant differences.

TABLE 1: General characteristics of patients with pituitary adenomas treated or not with statins.

	ACTHomas				GHomas				NFPTs			
General characteristics	**Total (42)**	**Patients with statins (14)**	**Patients without statins (28)**	***p*[a]**	**Total (28)**	**Patients with statins (6)**	**Patients without statins (22)**	***p*[a]**	**Total (62)**	**Patients with statins (12)**	**Patients without statins (36)**	***p*[a]**
Sex												
Male	4.8 (2/42)	7.1 (1/2)	3.6 (1/2)	ns	39.3 (11/28)	33.3 (2/11)	40.9 (9/11)	ns	54.8 (34/62)	26.9 (7/12)	73.1 (17/36)	ns
Female	95.2 (40/42)	92.6 (13/14)	96.4 (27/28)		60.7 (17/28)	66.7 (4/17)	59.1 (13/17)			22.7 (5/12)	77.3 (19/36)	
Age (years)	45 ± 2.07	53.6 ± 3.5	39.6 ± 2.7	0.003					56 ± 13.81	57 ± 5.0	56 ± 3.8	ns
BMI (Kg/m^2)	44.9 ± 12.1 (35/42)	31.1 ±1.85 (10/14)	32.6 ± 1.6 (19/28)	ns	28.76 ± 1.06 (15/28)	28.3 ± 1.2 (3/6)	28,9 ± 1.3 (12/22)	ns	28.1 ± 4.62 (21/62)	30.9 ± 3.2	27.6 ± 1.3	ns
Tumor												
Macroadenoma	45.2 (19/42)	71.4 (10/14)	32.1 (9/28)	ns	95.8 (23/24)	100 (4/4)	95.0 (19/20)	ns	100.0 (60/60)	100.0 (12/12)	100.0 (36/36)	ns
Microadenoma	42.9 (18/42)	14.3 (2/14)	57.1 (16/28)		4.2 (1/24)	0 (0/4)	5 (1/20)		0.0 (0/60)	0.0 (0/12)	0.0 (0/36)	
Not visible at MRI	11.9 (5/42)	14.3 (2/14)	10.7 (3/28)		0.0 (0/24)	0 (0/4)	0 (0/20)		0.0 (0/60)	0.0 (0/12)	0.0 (0/36)	
Tumor size (mm)	11.5 ± 1.41	16.2 ± 3.6	9.1 ± 1.4	ns	17.9 ± 1.3	18.9 ± 2.7	17.7 ±1.5	ns	20.8 ± 9.41	18.7 ± 6.2	27.5 ± 2.9	ns
Cephalea	11.8 (4/34)	25 (3/12)	4.5 (1/22)	ns	50 (14/28)	50(3/6)	50 (11/22)	ns	38.7 (24/62)	25.0 (5/12)	75 (15/36)	ns
Visual alterations	2.6 (1/38)	0 (0/14)	4.2 (1/24)	ns	16.7 (4/28)	0 (0/6)	18.2 (4/22)	ns	48.3 (28/58)	31.6 (6/12)	68.4 (13/32)	ns
Sinus invasion	-	-	-	-	52.0 (13/25)	5 (2/4)	52.4 (11/21)	ns	51.06 (24/47)	35.7 (5/12)	64.3 (9/23)	ns
Extrasellar growth	24.4 (10/41)	100 (1/1)	0 (0/1)	ns	72 (18/25)	75 (3/4)	71.4. (15/21)	ns	89.4 (42/47)	24.1 (7/12)	75.9 (22/23)	0.07
Pre-surgery treatment												
Ketoconazole	86.3 (33/38)	85.7 (12/14)	85.7 (21/24)	ns								
SSA	0.0 (0/42)	0.0 (0/14)	0.0 (0/28)	ns	0.0 (0/27)	0.0 (0/6)	0.0 (0/22)	ns	0.0 (0/55)	0.0 (0/12)	0.0 (0/36)	ns
Cabergoline	14.3 (6/42)	14.3 (2/14)	14.3 (4/28)		33.3 (9/27)	50 (3/6)	23.8 (5/21)	ns	0.0 (0/55)	0.0 (0/12)	0.0 (0/36)	ns
SSA + cabergoline	0.0 (0/42)	0.0 (0/14)	0.0 (0/28)	ns	3.7 (1/27)	0.0 (0/6)	4.8 (1/21)	ns	9.1 (5/55)	33.3 (1/12)	66.7 (2/36)	ns
				ns	29.6 (8/27)	33.3 (2/6)	19 (4/21)	ns	0.0 (0/55)	0.0 (0/12)	0.0 (0/36)	ns
ACTH at baseline	81.2± 9,5	78.4 ±22.5	97.5 ± 14.1	ns	-	-	-	-	-	-	-	-
GH at baseline	-	-	-	-	8.3 ± 1.52 (27/28)	14.1 ± 6.2 (3/6)	9.3 ± 2.6 (12/22)	ns	-	-	-	-
IGF1 at baseline	-	-	-	-	637.0 ± 53.36 (28/28)	766.8 ± 215.5 (3/6)	554.0 ±66.0 (12/22)	ns	-	-	-	-
Curation	52.4 (22/42)	57.1 (8/14)	60.7 (17/28)	ns	33.3 (9/27)	60 (3/5)	27.3 (6/22)	ns	25.0 (15/60)	10 (1/12)	90 (9/53)	ns
Biochemical control	84.6 (33/39)	84.6 (11/13)	84.6 (22/26)	ns	74.1 (20/27)	60 (3/5)	77.3 (17/22)	ns	-	-	-	-
Persistence	50.0 (20/40)	50.0 (4/8)	50.0 (16/32)	ns	-	-	-	-	78.3 (47/60)	85.7 (6/7)	77.4 (41/53)	-

Data given as % (n°/total) or mean ± SD, unless otherwise indicated.

[a]For the comparison between patients treated with statins with those not treated with statins.

Table 2: Results from free cytosolic calcium dynamic assays in PitNET cells in response to simvastatin.

Tumor type	# samples	Cells analyzed	% PRC	% PRM ± SEM	Time (s) ± SEM
ACTHomas	3/4	147	11 %	25 ± 2.9	16 ± 3.2
GHomas	0/5	155	0 %	-	-
NFPTs	2/3	93	9 %	20 ± 0.34	6.25 ± 0.89

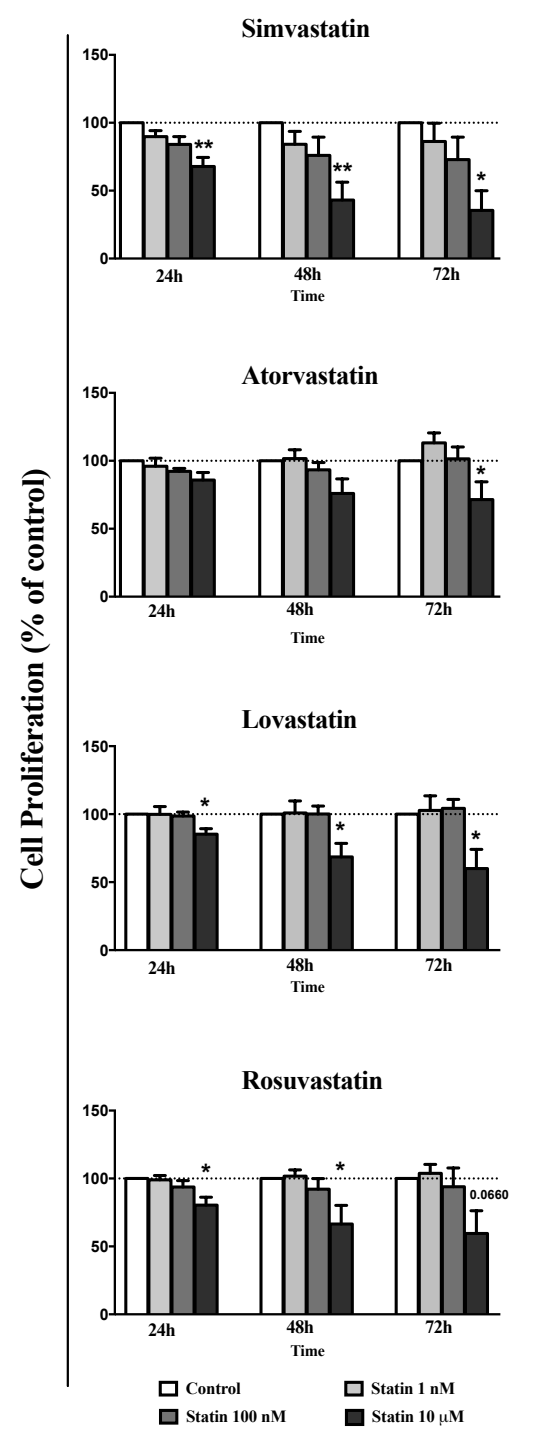
samples: number of responsive samples of the total of samples analyzed.

Cells analyzed: total of individual cells analyzed.

% PRC: percentage of responsive cells in responsive samples.

% PRM: percentage of maximum calcium reduction in response to simvastatin

Time: time of response to simvastatin administration.


Simvastatin
150
100
50
0
**
**
*
24h
48h
72h
Time
Atorvastatin
150
100
50
0
*
24h
48h
72h
Time
Lovastatin
150
100
50
0
*
*
*
24h
48h
72h
Time
Rosuvastatin
150
100
50
0
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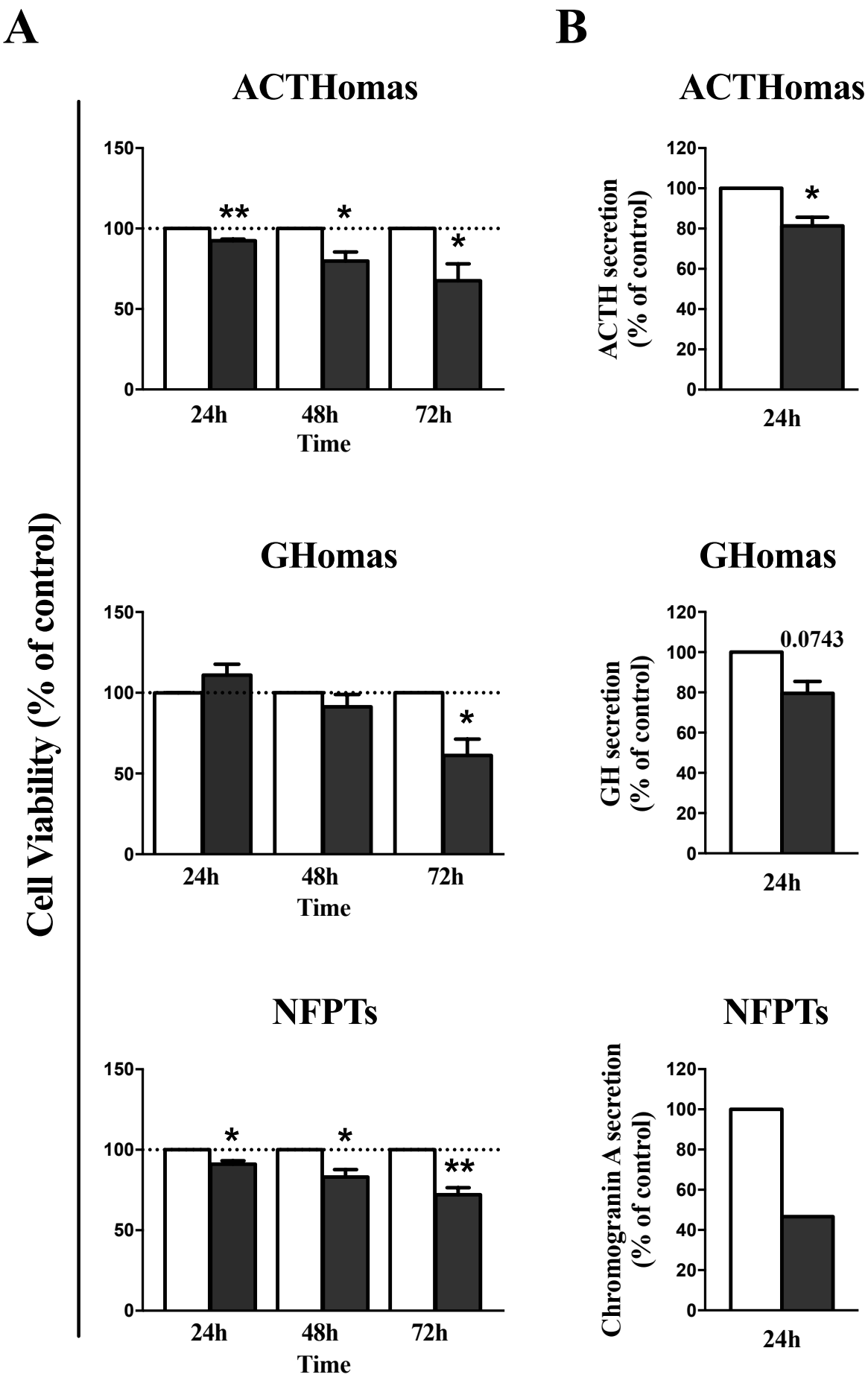
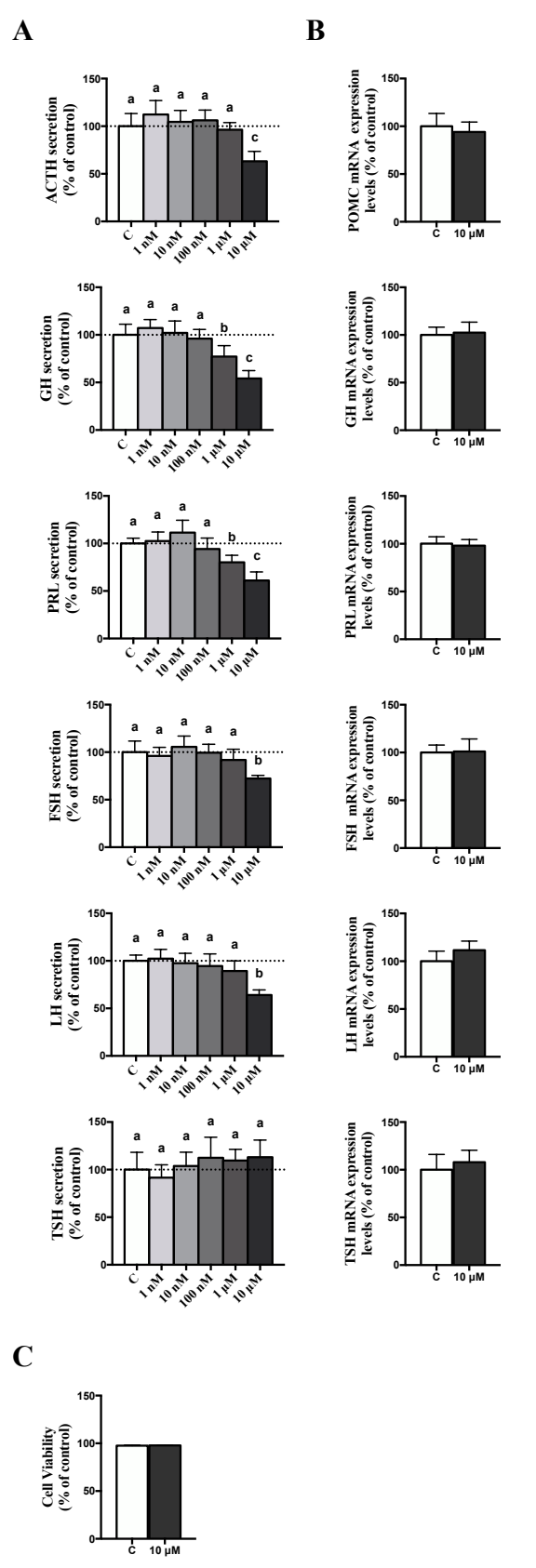
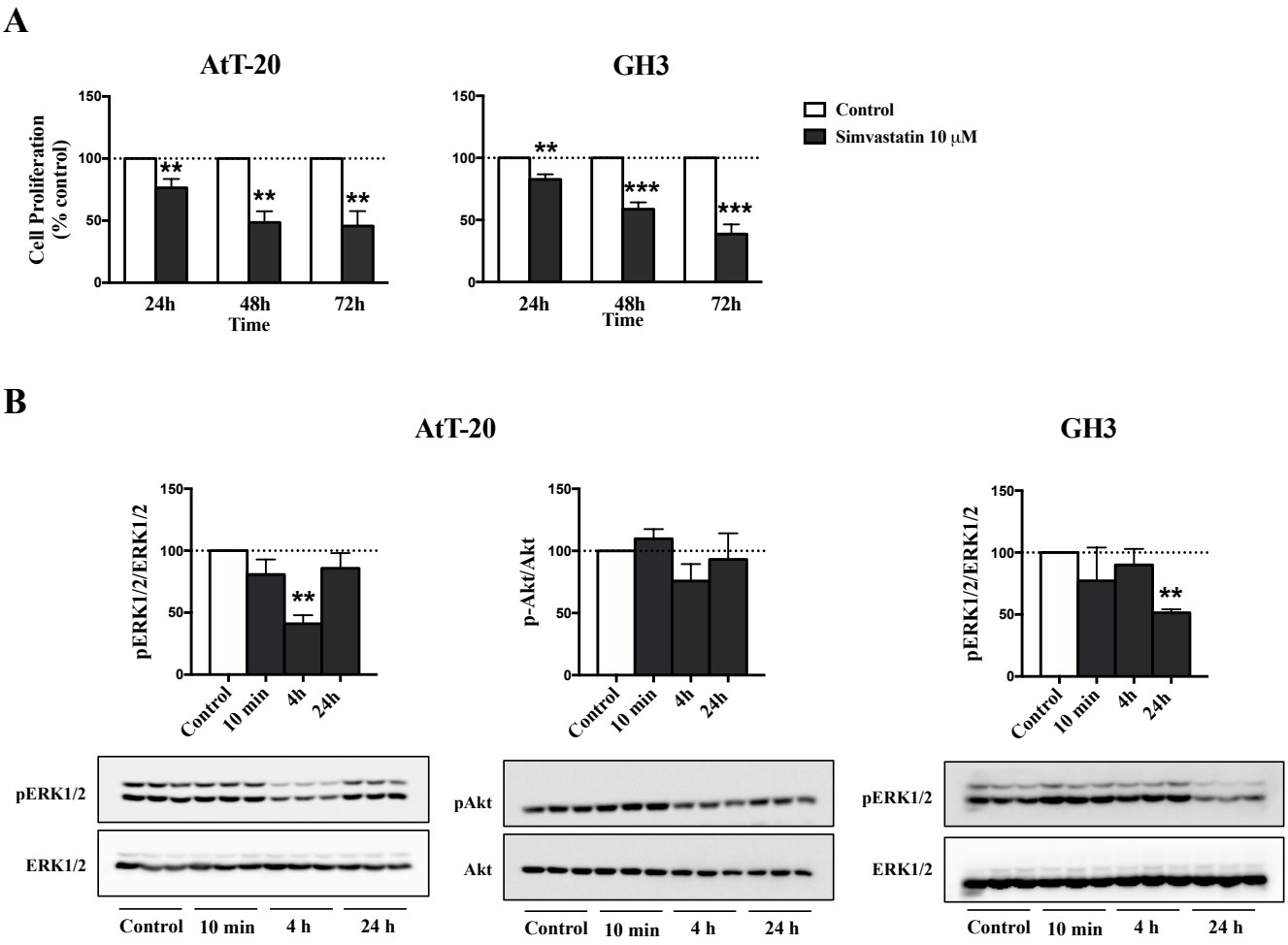
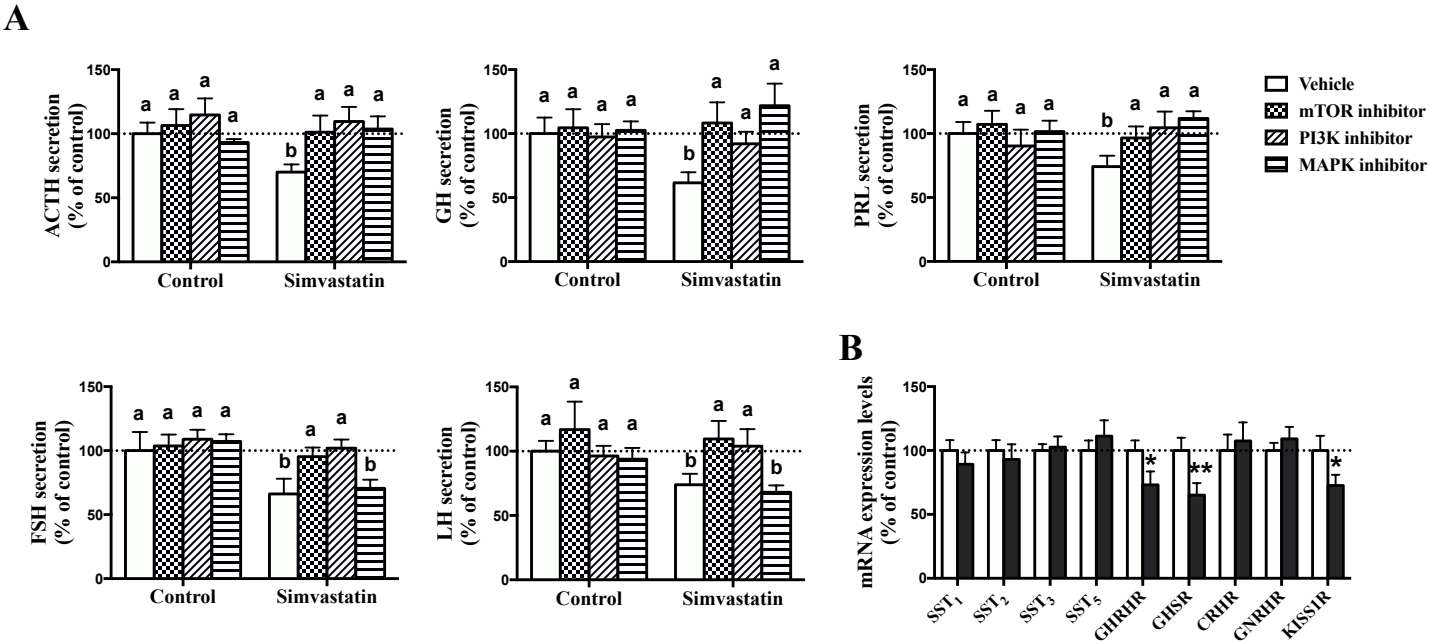
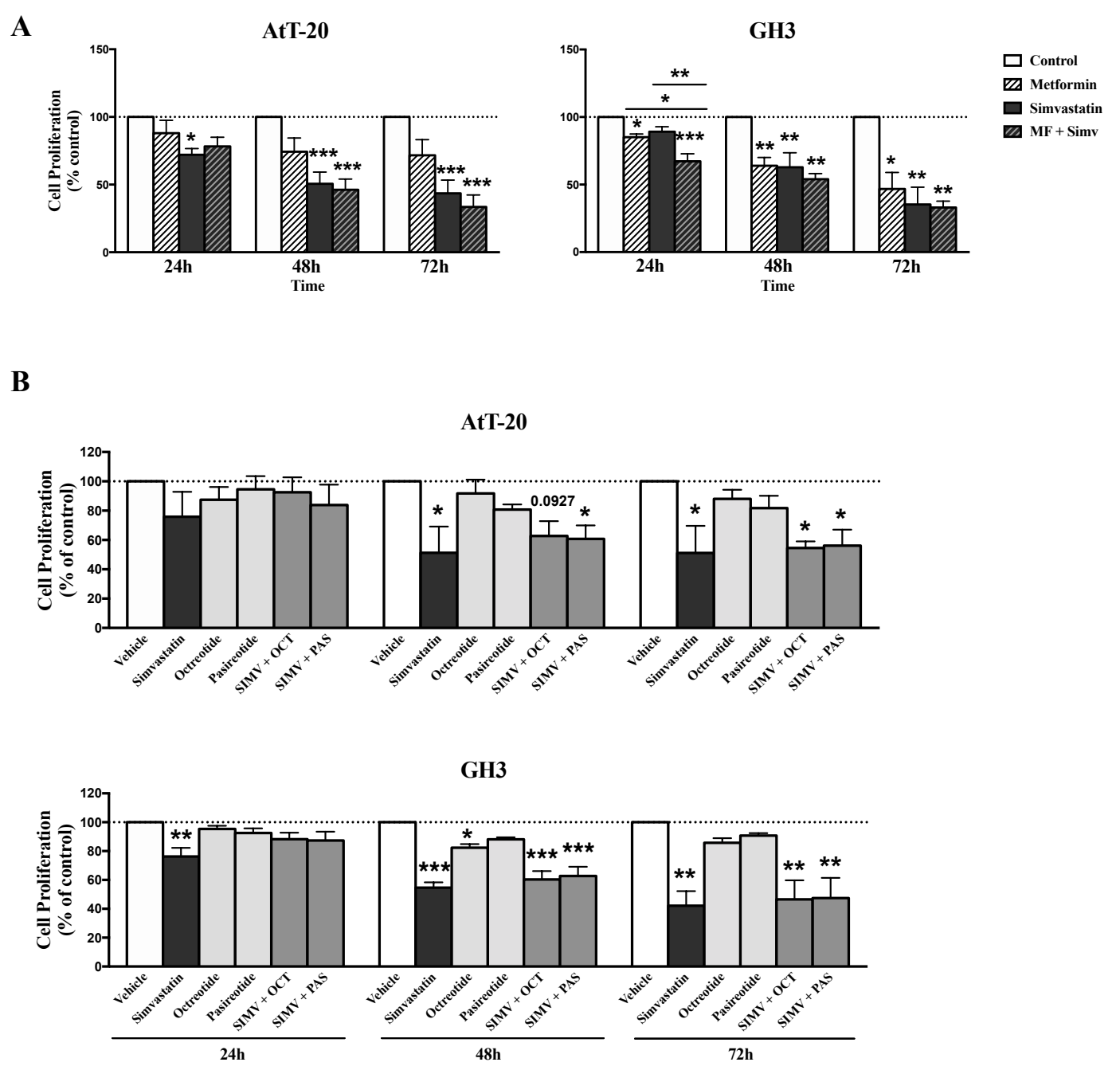
*
0.0660
24h
48h
72h
Time
Cell Proliferation (% of control)
Control
Statin 1 nM
Statin 100 nM
Statin 10 μM


A
B
ACTHomas
ACTHomas
Cell Viability (% of control)
150
100
50
0
**
*
*
24h
48h
72h
Time
ACTH secretion (% of control)
120
100
80
60
40
20
0
*
24h
GHomas
GHomas
150
100
50
0
*
24h
48h
72h
Time
GH secretion (% of control)
120
100
80
60
40
20
0
0.0743
24h
NFPTs
NFPTs
150
100
50
0
*
*
**
24h
48h
72h
Time
Chromogranin A secretion (% of control)
120
100
80
60
40
20
0
24h
Control
Simvastatin


A
B
ACTH secretion (% of control)
POMC mRNA expression levels (% of control)
GH secretion (% of control)
GH mRNA expression levels (% of control)
PRL secretion (% of control)
PRL mRNA expression levels (% of control)
FSH secretion (% of control)
FSH mRNA expression levels (% of control)
LH secretion (% of control)
LH mRNA expression levels (% of control)
TSH secretion (% of control)
TSH mRNA expression levels (% of control)
C
1 nM
10 nM
100 nM
1 µM
10 µM
10 µM
C
Cell Viability (% of control)


A
AtT-20
GH3
Cell Proliferation (% control)
150
100
50
0
**
***
24h
48h
72h
Time
Control
Simvastatin 10 μM
B
AtT-20
GH3
pERK1/2/ERK1/2
p-Akt/Akt
Control
10 min
4h
24h
pERK1/2
ERK1/2
pAkt
Akt
4 h
24 h


A
ACTH secretion (% of control)
GH secretion (% of control)
PRL secretion (% of control)
FSH secretion (% of control)
LH secretion (% of control)
Control
Simvastatin
Vehicle
mTOR inhibitor
PI3K inhibitor
MAPK inhibitor
B
mRNA expression levels (% of control)
SST1
SST2
SST3
SST5
GHRHR
GHSR
CRHR
GNRHR
KISS1R


A
AtT-20
GH3
Control
Metformin
Simvastatin
MF + Simv
Cell Proliferation (% control)
150
100
50
0
24h
48h
72h
Time
B
AtT-20
GH3
Cell Proliferation (% of control)
120
100
80
60
40
20
0
0.0927
Vehicle
Simvastatin
Octreotide
Pasireotide
SIMV + OCT
SIMV + PAS
24h
48h
72h

A novel SST_3 agonist shows potential antitumor effects in experimental models of Nonfunctioning Pituitary Tumors

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ABSTRACT

Somatostatin-analogs (SSAs) are efficacious and safe treatments for a variety of neuroendocrine tumors, especially pituitary neuroendocrine tumors (PitNETs). Their therapeutic effects are mainly mediated by somatostatin receptors SST_2 and SST_5. Most peptidic SSAs, such as Octreotide/Lanreotide/Pasireotide, are either nonselective or activate mainly SST_2. However, non-functioning pituitary tumors (NFPTs), the most common PitNET type, mainly express SST_3 and finding peptides that activate this particular somatostatin receptor has been very challenging. Therefore, the main objective of this study was to identify SST_3-agonists and characterize their effects on experimental NFPT models. Binding to SSTs and cAMP level determinations were used to screen a peptide library and identify SST_3-agonists. Key functional parameters (cell viability, caspase activity, chromogranin A secretion, mRNA expression and intracellular signaling pathways) were assessed on NFPT primary cell cultures in response to SST_3-agonists. Tumor growth was assessed in a preclinical PitNET mouse model treated with a SST_3-agonist. Thus, we successfully identified the first SST_3-agonist peptides. SST_3-agonists lowered cell viability and chromogranin A secretion, increased apoptosis *in vitro*, and reduced tumor growth in a preclinical PitNET model. As expected, inhibition of cell viability in response to SST_3-agonists defined two NFPTs populations: responsive and unresponsive, wherein responsive NFPTs expressed more SST_3 than unresponsive NFPTs and exhibited a profound reduction of MAPK, PI3K-AKT/mTOR and JAK/STAT signaling-pathways upon SST_3-agonist treatments. Concurrently, *SSTR3*-silencing increased cell viability in a subset of NFPTs. In conclusion, this study demonstrates that SST_3-agonists activate signaling mechanisms that reduce NFPT cell viability and inhibit pituitary tumor growth in experimental models that expresses SST_3, suggesting that targeting this receptor could be an efficacious treatment for NFPTs.

KEYWORDS: SST_3 / non-functioning pituitary tumors / agonists / antagonist / primary cultures.

INTRODUCTION

Somatostatin receptors (SST_{1-5}) comprise a family of seven transmembrane G-protein coupled receptors able to bind and be activated by somatostatin [41,3]. SSTs activation has been widely associated to multiple effects, most of them inhibitory actions on hormone secretion and cellular processes such as proliferation in normal and tumor tissues [41]. Thus, SSTs are considered as an attractive therapeutic target to treat different tumor pathologies [41,43]. Clinically available somatostatin-analogs (Octreotide/Lanreotide/Pasireotide) are used to treat neuroendocrine tumors (NETs), including pituitary neuroendocrine tumors (PitNETs), where they reduce/normalize hormonal levels, shrink tumors, and improve clinical symptoms [41,31]. Most of their therapeutic actions are assumed to be mediated through SST_2 and SST_5 activation [41]. Although SSAs are able to bind other SSTs, such as SST_3, this binding capacity is significantly lower in comparison to SST_2/SST_5 [41]. Remarkably, some NETs types express SST_3 at higher or similar levels than SST_2 or SST_5. This is the case for some gastroenteropancreatic NETs [15], pheochromocytomas [9] and specially non-functioning pituitary tumors (NFPTs), which represent a heterogeneous group of tumors that constitute 30% of all PitNETs [19,16,25,2].

Most NFPTs are silent gonadotropinomas characterized by lack of hormone hypersecretion, which usually determines a delay in diagnosis [2] and, therefore, are mostly detected as macroadenomas with consequently associated severe comorbidities related to mass effect (i.e. headaches/visual defects/hypopituitarism) [12,11]. Transsphenoidal surgery is the first-line therapy and the only current curative approach of NFPTs; however, is often not definitive due to the invasion of surrounding structures (sphenoidal/cavernous sinuses) and tumor relapses are frequent even when resection seems total (20-30% of relapses at 10 years after the first surgery) [32,6]. Radiotherapy has been largely used to prevent tumor relapses but long-term side effects have been reported [20,34]. To date, the pharmacological treatment options for NFPTs are insufficient. Unfortunately, drugs currently available for functional PitNETs, such as dopamine agonists (DAs) or SSAs, have shown poor efficacy in NFPTs [22,34]. Moreover, *in vitro* treatment of NFPT primary cell cultures with octreotide may not decrease cell viability [19], which could be explained by the low SST_2/SST_5 expression levels [16,25], as mentioned above.

In this scenario, the importance of SST_3 relies on the fact that SST_3 has been associated with apoptotic/antiproliferative actions in various studies using cell lines [41,45,40]. Indeed, the apoptotic actions of somatostatin/SSAs have been historically related to SST_3 [41,45,40]. Naturally, pasireotide has been suggested

as a potential therapeutic option due to its ability to bind to SST_3 with higher affinity than octreotide/lanreotide [41,25]. However, recent results from our group demonstrate that it does not have a clear inhibitory effect on NFPT primary cultures, being even less potent than octreotide [19], thus reinforcing the notion that identification and validation of novel therapeutic approaches is necessary to manage NFPTs. Hence, based on all this information, we hypothesized that a compound that preferentially binds to SST_3 might be an effective therapeutic option to treat NFPTs. However, it has been very challenging to find peptides that selectively activate SST_3, and its functional role and pathophysiological relevance in NFPTs still need further demonstration. Therefore, the aims of this study were to: 1) identify specific SST_3-agonists through the screening of a peptide library; and 2) determine the *in vitro/in vivo* therapeutic potential of SST_3 by studying: 2A) the direct effect of the identified SST_3-specific agonists/antagonists on key functional parameters for NFPTs (i.e. cell viability/caspase activity/chromogranin A secretion/signaling pathways/mRNA expression) and the functional consequences of *SSTR3* silencing on cell viability; and, 2B) the effect of a selected SST_3-specific agonist on tumor growth in a preclinical mouse model of PitNETs.

MATERIALS AND METHODS

Reagents

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. Subtype selective SST_3 agonists (L-796,778/BIM-355/BIM-071) and antagonists (BIM-839/BIM-152, previously known as BN81658), were generously provided by Merck & Co., Inc. (L-796,778; Whitehouse Station, NJ, USA) or IPSEN Bioscience, Inc. (BIM-compounds; Cambridge, MA, USA). Specificity of the MERCK human-agonist was previously reported [36]. Detailed information of the affinity profile [expressed as Ki (nM)] and structure of the human SST_3 agonists/antagonists is described in Supplementary Table-S1 and Fig-1.

Library screening

The identification of compounds that act as selective SST_3-agonists was based on the examination of IPSEN's extensive collection of synthetic peptides: see supplemental information for details.

Radioligand binding assays

The affinities of the compounds of interest for the different human and mouse SSTs were determined by radioligand binding assays in HEK293 cells (ATCC) stably transfected with each receptor. Single clones were selected based on their mRNA expression measured by qPCR, further selected by their membrane expression of each SST, and by their response to lanreotide in cAMP assays. Membranes were isolated from these cells, mixed with trace concentrations of each specific radioligand and a range of concentrations of the compounds of interest, and then incubated at room temperature for 30-60 minutes. Unbound radioligands were removed by filtration through GF/C filters and membrane-bound radioactivity was measured using a gamma counter. Nonspecific binding was defined in the presence of 1 µM lanreotide. The inhibition constants (K_i) were calculated from the following equation: $K_i = IC_{50}/(1+L/K_d)$ where L is the radioligand concentration and K_d the equilibrium dissociation constant of the radioligand for each receptor subtype. Radioligands were: [125I-Tyr11]-lanreotide for SST_3, [125I-Tyr]-seglitide for SST_2 and [125I-Tyr]-BIM-23015 for SST_5.

Cyclic AMP accumulation assay

The functional agonist activities of the compounds of interest in inhibiting intracellular cAMP production (stimulated by forskolin) were determined in the same cell lines used for radioligand binding cells (HEK293 cells stably expressing different human and mouse SSTs). Cells were plated in 384-well plates and cultured for 16-24 hours at 37°C. Compounds of interest at a range of concentrations were incubated with the cells in the presence of forskolin for 30 minutes. Cell homogenates were incubated with fluorescence-labelled cAMP and anti-cAMP antibodies for 1 hour, using the cAMP Dynamic 2 kit (Cisbio Bioassays), before reading the homogeneous time resolved fluorescence.

Patients, samples and primary cell cultures

Three different types of pituitary samples have been included in the present study: Cohort 1) Fresh samples from 80 NFPTs (mean age: 57 [18-80] years; 42% women) and 80 functioning PitNETs [63 GHomas (mean age: 43 [12-73] years; 48% women) and 17 ACTHomas (mean age: 38 [20-67] years; 94% women)] obtained during transsphenoidal surgery in different hospitals from Spain [formalin–fixed paraffin-embedded (FFPE) pieces were also available for n=29 NFPT samples]; Cohort 2) 35 FFPE NFPTs [n=25 tumors from France (Hospices Civils de Lyon, Lyon) and n=10 from Spain (Hospital Universitario Virgen del Rocío, Seville)] (mean age: 63 [25-82] years; 46% women); and Cohort 3) 12 normal pituitary (NP) samples (mean age: 61 [44-85; 50% women] obtained during autopsies. All techniques carried out in this study were conducted in accordance with the ethical standards of the Helsinki Declaration, of the World Medical Association and with

the approval of the University of Cordoba/IMIBIC and Ethics Committees from all the Hospitals involved in the study. Informed consent from each patient or relative, in case of autopsy, was obtained. See supplemental information for details.

RNA isolation, reverse transcription and analysis of gene expression levels by qPCR

Information about RNA extraction, quantification, reverse-transcription (RT) and qPCR using specific primers included in this study has been previously reported elsewhere by our group [18,1]. See supplemental information for more details.

Immunohistochemical analysis (IHC) for SST_3 in NFPTs

IHC staining for SST_3 was performed using an automated immunostainer (Benchmark XT, Ventana Medical Systems, Tucson, AZ) to corroborate the presence of SST_3 in a set of 35 FFPE NFPTs [Cohort 2: n=25 tumors from France (Hospices Civils de Lyon, Lyon) and n=10 from Spain (Hospital Universitario Virgen del Rocío, Seville)] and to evaluate the presence of SST_3 in SST_3-agonist responsive (n=17) and unresponsive (n=12) NFPTs (Available FFPE samples from Cohort 1). See supplemental information for details.

Measurement of cell viability in primary pituitary tumor cell cultures

Viability of primary pituitary tumor cells was evaluated every 24h (10,000 cells/well in 96-well plates) in response to SST_3-agonists and antagonists (treatments were daily refreshed after each measure) using Alamar-blue reagent (Invitrogen, Madrid, Spain) and a FlexStation III system (Molecular Devices, Sunnyvale, CA), as previously reported [18].

Measurement of caspase activity of primary pituitary tumor cell cultures

As a surrogate marker of apoptosis induction capacity, we measured caspase 3/7 activity in response to different SST_3-agonists and BIM-839 antagonist using the Caspase-Glo 3/7 assay (Promega, Madrid, Spain) according to manufacturer´s instructions. See supplemental information for details.

Measurement of chromogranin A release in primary pituitary tumor cell cultures

To evaluate the effects of SST_3-agonists on chromogranin-A (CgA) release, 150,000-200,000 cells/well were used. Media were recollected after 24h of treatment and CgA concentration was evaluated using a commercial ELISA (EIA-4937; DRG, Mountainside, NJ, USA).

Measurement of free cytosolic calcium concentration ($[Ca^{2+}]_i$) kinetics

To assess the effects of different SST_3-agonists on free cytosolic calcium kinetics, 50,000 cells/coverslip were plated and changes in $[Ca^{2+}]_i$ in single cells were measured using fura-2AM (Molecular Probes, Eugene, OR), as described previously [29,18].

Analysis of signaling pathways by human phospho-kinase array.

To test the signaling pathways altered in responsive and unresponsive NFPTs treated with a selected SST_3-agonist, 500,000 cells/well were cultured in 12-well plates and incubated for 8 minutes with vehicle and BIM-355. See supplemental information for details.

Preclinical mouse model

A preclinical mouse model of non-secreting PitNET (the POMC-knockout generated through homologous gene targeting in embryonic stem cells) was used as previously reported [21,47]. Four-weeks after birth, mice were treated with vehicle or a selected SST_3-agonist by subcutaneous implantation of 7-day mini-pumps (ALZET) for eight weeks. Three groups were established: vehicle (n=16), low BIM-355 dose (2.2 mg/kg/day; n=8) and high BIM-355 dose (7.4 mg/kg/day; n=6). Tumor volume was measured weekly by MRI-imaging. After euthanasia, pituitary glands were carefully removed, fixed and embedded in paraffin. IHC for SST_3 and Ki67 were performed in the mouse pituitary samples. All experimental procedures were carried out following the European Regulations for Animal Care, in accordance with guidelines and regulations, and under the approval of the University/Regional's Government Research Ethics Committees.

Silencing of SSTR3 gene with specific siRNAs

To perform silencing experiments, 500,000 cells/well were plated and transfected with two specific *SSTR3* siRNAs at 100nM (s13501 and s224690 Silencer Select Pre-designed siRNAs; Ambion, Wilmington, NC) and a commercial negative control (Scramble: Silencer Select Negative Control 1 siRNA; Ambion) using Lipofectamine RNAiMAX (Life Technologies). See supplemental information for details.

Statistical analysis and receiver operating characteristic (ROC) curves

All data are expressed as mean ± SEM. Statistical differences between the expression levels of NFPT and NPs were assessed by unpaired nonparametric Mann-Whitney tests (according to normality evaluated by Kolmogorov-Smirnov test). Differences between SSTs mRNA levels within NFPTs or, comparison of SST_3 levels between NFPT, functioning PitNETs and NPs were assessed by Kruskal-Wallis test followed by Dunn's

test for multiple comparisons. As previously reported [29], to normalize values within each treatment and minimize intragroup variations in the different *in vitro* experiments (i.e. different age of the tissue donor or metabolic environment), the values obtained were compared with vehicle-treated controls (set at 100%). All experiments were performed in a minimum of three independent primary pituitary cultures from different patients (3-4 replicates/treatment per experiment), unless otherwise indicated. $p<0.05$ was considered significant. Regarding the preclinical model, a generalized linear mixed model to account for repeated measures was used to evaluate tumor volume across time (baseline to week 8) adjusted by baseline tumor volume, time, treatment group, interaction between time and treatment group, and interaction between baseline tumor volume and time. The log-normal distribution analysed by SAS 9.4 was found to be the best fit. The results were expressed as geometric least square (LS) means for tumor volume across time from baseline to week 8 for the different treatment groups. In addition, geometric LS adjusted for baseline tumor volume was analysed for each treatment group by time and was adjusted by Tukey-Kram.

As previously reported [33,30], ROC-curves were used as a tool to measure how well the expression of SST_3 could discriminate between different diagnostic groups. Statistical analysis of ROC curves was performed by calculating the Area Under the Curve (AUC) of each receptor and comparing them with the AUC of the reference line using Student's t-test. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software; La Jolla, CA, USA).

For more details of methods see Supplementary information.

RESULTS

Identification of SST_3 peptide agonists

We screened a library of synthetic peptides produced at IPSEN, first selecting for compounds that bind to SST_3 and then applying a multidimensional Spotfire® analysis designed to identify compounds with the highest selectivity for SST_3 versus SST_2 and SST_5. This tool was customized to display the selectivity of every compound for each of the three SSTs tested along three different axes. The SST_3 selective compounds showed a clearly different localization in this three-dimensional space clustering together on the SST_3 axis. The peptides obtained in the Sporfire analysis (i.e. BIM-23A185), while being active in receptor assays, were only hit compounds and did not have optimal drug-like properties. For example, their solubility and stability were suboptimal in standard quality control measurements. For this reason, small structural changes were introduced

into the structure of BIM-23A185 to obtain derivates with more drug-like characteristics. These changes yielded several peptides (i.e. BIM-355, BIM-071, BIM-839 among others) that preserved the general similarity with BIM-23A185 and had improved solubility, stability, reproducible binding, and pharmacological activity. Several batches of these compounds were synthesized independently and tested to confirm their structures and activities. High-throughput synthesis of these peptides allowed the characterization of their binding, selectivity, and functional activity on different SSTs (Table-S1), being BIM-23A185 discarded for further analyses due to the low level of robustness of the results generated (data not shown). The most potent peptides (BIM-355, BIM-071, BIM-839) bind to SST_3 with affinities in the low nanomolar range while the binding to SST_2 and SST_5 is orders of magnitude weaker than Octreotide/Lanreotide/Pasireotide [3]. Functional cAMP assays confirmed that the newly identified peptides (BIM-355, BIM-071) activate SST_3, while BIM-839 showed SST_3-antagonist activity. Based on these results, we selected BIM-355, BIM-071, and BIM-839 as the most selective and potent compounds for further characterization. Binding-assays using human/rat/mouse SST_3 showed that BIM-071 does not activate rodent receptors. Based on all these results we selected these peptides to perform experiments in human NFPT primary cultures and *in vivo* in mice with PitNETs.

SST_3-agonists modulate key functional parameters in NFPT primary cell cultures

SST_3 was the highest expressed receptor in our cohort of NFPTs followed by SST_2, SST_1, SST_5 and the two SST_5 splicing-variants (SST_5TMD4/SST_5TMD5) (Fig-S1A-C). We also found a clear differential SST_3 expression pattern between functioning PitNETs (GHomas/ACTHomas) and NFPTs, being this expression significantly lower in GHomas/ACTHomas vs. NFPTs (Fig-S2A-D). Moreover, SST_3 IHC-staining was present in approximately 50% of samples, showing in all cases similar membrane and cytoplasmic localizations (Fig-S1D), as previously reported [25,28]. We next explored the functional role of SST_3 using the new peptidic SST_3-agonists/antagonists in comparison to the previously published non-peptidic L-796,778 SST_3-agonist. All SST_3-agonists induced a clear concentration-dependent reduction of cell viability, being 10^{-7}M, the lowest concentration that caused a maximal effect, selected for further experiments (Fig-S3A). The antagonists BIM-839 and BIM-152 did not elicit changes on cell viability at any of the concentration tested (10^{-5} to 10^{-11}M and 10^{-6} to 10^{-11}, respectively; Fig-S3B).

When comparing the effects of all SST_3-agonists (at 10^{-7}M), we observed a comparable significant decrease of cell viability after 48-72h of incubation (23.2%, 18.3% and 17.9% of reduction at 72h with BIM-355, BIM-071 and L-796,778, respectively), although BIM-355 seemed to be the agonist exerting a stronger

reduction (Fig-2A). To demonstrate the specificity of these effects, we blocked them with two antagonists. Co-administration of BIM-839 (10^{-5}M) or BIM-152 (10^{-9}M) with each agonist (10^{-7}M) fully counteracted the agonist´s effect, confirming the functional role of SST_3 in these effects (Fig-2B). Remarkably, at the selected concentrations, BIM-839 did not alter cell viability (Fig-2B) but BIM-152 reduced cell viability at high concentrations in some tumors, which prompted us not to use this compound in subsequent experiments. Additionally, administration of BIM-355, BIM-071 or L-796,778 increased caspase 3/7 activity (indicator of an increase on apoptotic rate), BIM-355 appearing as the most potent compound. Of note, these effects were also completely blocked in the presence of BIM-839 (Fig-2C).

Furthermore, BIM-355, BIM-071 and L-796,778 clearly reduced CgA-secretion (considered as a valuable marker of NFPTs [14,50]) after 24h incubation (Fig-2D). Finally, we analyzed whether SST_3-agonists may regulate the expression of key genes related with the pathogenesis of PitNETs [13] (Fig. 2E). Incubation of BIM-355, but not BIM-071, reduced gonadotropin-hormones expression (*FSHB* and *LHB*), whereas BIM-071 significantly reduced the common α-subunit (*CGA*) mRNA levels. However, incubation with L-796,778 did not alter *CGA*, *FSHB* or *LHB* levels. Moreover, *PTTG1*, and *MYC* levels were not altered in response to the different agonists but *TP53* was significantly decreased in response to BIM-355 (Fig-2E).

Although these data demonstrate important roles of SST_3 in NFPT cells, it should be noted that a proportion of the NFPTs analyzed did not respond to SST_3-agonists. This conclusion is mainly based on the study of cell viability, which was evaluated in all the available NFPT cell cultures. Indeed, 16 out of the 42 NFPT cultures tested (38%) did not show a reduction of cell viability in response to any of the SST_3-agonists tested (at least two SST_3-agonists were tested per NFPT culture). The functional assays performed in each individual responsive and unresponsive NFPT and the responsiveness to each SST_3-agonist is shown on Table S2 and S3.

Differential SST_3 expression in responsive *vs.* unresponsive NFPTs

To understand the differential response to SST_3-agonists observed in cell viability assays, we compared the SSTs expression levels between responsive and unresponsive tumors. Interestingly, we found higher SST_3 mRNA and protein levels (statistically significant changes: p=0.029 and 0.039, respectively) in responsive vs. unresponsive tumors (Fig-3A-C). However, no significant changes were found in the expression of other SST-subtypes between the two NFPT-populations at mRNA (Fig-3A). Moreover, while SST_1/SST_2/SST_5/SST_5TMD4/SST_5TMD5 mRNA levels were unable to distinguish between both groups, SST_3

expression levels significantly discriminated between responsive and unresponsive tumors (mRNA levels: AUC=0.71; p=0.03; Fig-3A and Fig-S4; and protein levels: AUC=0.69; p=0.098; Fig-3B). We also evaluated if any clinical parameter (extrasellar growth, cavernous sinus invasion, cure rate, etc) could be different, and able to discriminate, between responsive and unresponsive tumors. However, Chi-square tests did not reveal any significant result (Table S4), suggesting that only the SST_3 expression pattern is able to discriminate between responsive vs. unresponsive NFPTs. Taking into account the differential SST_3 expression pattern, we further investigated the capacity of different SST_3-agonists to modulate this receptor expression profile in both responsive and unresponsive tumors. This revealed that BIM-355 and L-796,778 clearly increased SST_3 mRNA levels (24h-incubation) in responsive primary NFPT-cultures, whereas SST_3 expression was reduced in unresponsive NFPTs in response to BIM-071, but unaltered in response to BIM-355 and L-796,778 (Fig-3D).

Signaling pathways modulated in response to SST_3-agonists

We next identified the signaling pathways underlying the ability of SST_3-agonists to generate functional responses. Specifically, NFPT cells showed little response to SST_3-agonists in terms of $[Ca^{2+}]_i$ kinetics (12.4, 10 and 7% of responsive cells to BIM-355, BIM-071 and L-796,778, respectively) with modest $[Ca^{2+}]_i$ reductions, ranging between 25.6 and 27.4% (Fig-S5A). Conversely, BIM-355 treated cells from responsive NFPTs showed a decrease in phosphorylated proteins levels involved in three major signaling-pathways: MAPK (HSP27/PLCG1/ERK1-2/RSK1-2-3/CREB/JNK1-2-3/c-Jun), PI3K-AKT/mTOR (Akt,/GSK-3a/b/Src/FAK/p53/PRAS40/TOR/p70 S6-kinase) and JAK/STAT (STAT3/STAT5a-b/STAT6), when compared to vehicle-treated controls (Fig-4A). In contrast, BIM-355 treatment of unresponsive NFPTs did not evoke similar reductions, and even elicited an increase of phosphorylated levels of several proteins belonging to the same pathways, such as MSK1-2/c-Jun/Src/FAK/TOR/STAT2/STAT5a-b/STAT5b/STAT6 (Fig-4B). Interestingly, we observed clearly reduced basal phosphorylation levels in most of these proteins in responsive NFPTs compared to unresponsive tumors (Fig-S5B).

Effect of *SSTR3* gene silencing on cell viability

SSTR3 gene silencing was achieved at the mRNA levels using two specific siRNAs (Fig-5A). Additionally, we could test the silencing at protein level in 2 independent NFPTs at 48- or 72h after transfection, respectively. This approach revealed a SST_3 content reduction of 70% (48h after transfection with s13501) and 20-40% (72h after transfection with s13501 and s224690 siRNA, respectively) compared to scramble-transfected cells in the two NFPTs analyzed (Fig-S6A-B). Particularly, s13501 did not alter cell viability, but

s224690 significantly increased cell viability 48-72h after transfection in 38% of NFPTs analyzed (those named responsive to silencing) compared to scramble-transfected cells (Fig-5B). This differential response to s224690 was not associated to the initial SST_3 levels (Fig-5C), to the percentage of reduction in the level of SST_3 (Fig-5D) or to the presence of the endogenous ligands of SST_3, somatostatin or cortistatin. Indeed, this analysis revealed low levels of somatostatin (average of 0.0066 mRNA copies adjusted by *HPRT*) and much higher levels of cortistatin (average of 0.98 mRNA copies adjusted by *HPRT*), with no significant differences between responders and non-responders to *SSTR3*-silencing (data not shown). On the other hand, and as expected, the treatment with BIM-355 induced a reduction on cell viability in scramble-transfected cells; however, this effect was completely blunted in s13501-transfected cells where the SST_3 content was clearly reduced (Fig-S6C). This approach was implemented in cells from a single NFPT and suggests that the effect of BIM-355 is mediated by SST_3.

SST_3-agonist reduced tumor growth in an *in vivo* preclinical model

To demonstrate the ability of SST_3 to mediate a reduction in tumor growth *in vivo*, we used the POMC-KO mice, a mouse model of PitNETs. Mice were treated with two doses of BIM-355 (group-1: 2.2mg/kg/day and group-2: 7.4mg/kg/day). Treatment with BIM-355 reduced tumor-size after 8-weeks of treatment in a dose-dependent manner. This reduction was statistically significant only at the higher dose (Fig-6C/D; it should be noted that although 6 animals started in the higher dose group, 3 of them were removed after three weeks due to an irritation at the minipump site. This can be clearly seen in Fig-S7. Additionally, IHC analysis of the tumors formed confirmed the presence of SST_3-staining (Fig-6A), and a strong presence of Ki67-staining in the tumor area in contrast to the normal/adjacent mice pituitary gland (Fig-6B).

DISCUSSION

In this study, we identified for the first time two selective peptidic SST_3-agonists and used them to explore the functional relevance of SST_3 in NFPTs using a battery of experimental and analytical techniques. Our first objective was to identify specific SST_3-agonists that could activate SST_3 through the screening of a peptide library. From all tested peptides, SST_3 binding affinities and functional cAMP assays revealed that BIM-355 and BIM-071 were the most potent and specific SST_3-agonists, while BIM-839 was the most potent SST_3-antagonist.

To determine whether SST_3 could play a significant pathophysiological role in NFPT cells, we first corroborated that SST_3 was the highest overexpressed receptor in our NFPT cohort, and demonstrated that SST_3 mRNA levels could discriminate between NFPTs vs. functioning PitNETs, but not between NPs vs. functioning PitNETs. Moreover, use of IHC also validated the presence of SST_3 protein in approximately 50% of NFPTs analyzed, using two different cohorts of patients, which presented both a membrane and cytoplasmic localization, as previously described [25]. To further explore the functional relevance of this receptor in NFPTs, we compared the effects of the two peptidic agonists identified herein with those exerted by the previously published non-peptidic agonist L-796,778 [36]. The first approach was to assess the effect of SST_3-agonists treatment on cell viability, a parameter tightly linked to tumor growth, the main clinical problem associated to NFPTs. Our data revealed a general ability of SST_3-agonists to reduce cell viability in NFPT cells cultured *in vitro*, wherein BIM-355 was the most potent agonist (i.e. 23.2% of reduction), which, to our knowledge, is the most prominent reduction reached to date in a cell culture model on cell viability through the activation of SST_3 [e.g. treatment with the SST_3-agonist (L-796,778) exerted a 19% inhibition on cell proliferation in HEK-293 cells in previous studies [45]]. At this point, it is important to note that pituitary tumor cells, and specially NFPT cells, are not as markedly responsive to pharmacological treatments as cells from other tumor types [19,18]. Most importantly, we also observed a clear increase of caspase activity, which is likely translated into an increase of apoptosis, in response to treatment with different SST_3-agonists. These effects on caspase activity, as well as those on cell viability, were completely blocked by the antagonist BIM-839, thus confirming the specificity of the actions observed. These results are in line with the previous results mentioned above, which indicated that treatment with L-796,778 increased the apoptotic index or related markers in the HEK-293 cell line [45]. Moreover, similar effects were reported in SST_3-overexpressing breast cancer cells [44], further suggesting a clear, robust antiproliferative and proapoptotic role of this receptor. Of note, in support of the anti-proliferative and proapoptotic role of SST_3 in NFPTs observed in the present study, we demonstrated, for the first time, that *in vivo* administration of an SST_3-specific agonist significantly decreased tumor growth in a preclinical mouse model of PitNET [47,21]. It has to be also noted that the differential response observed in the functional assays depending on the SST_3-agonist used may be due to the highly complex activation of G protein-coupled receptors (GPCRs), including the presence of multiple ligand-binding sites that can influence signal transduction in a distinct manner [23].

Our data also revealed a significant decrease of CgA secretion/expression in NFPT cells cultured *in vitro* in response to SST_3-agonists. In addition to CGA, a reduction of FSH/LH mRNA was found in response to

BIM-355 (the most effective compound), which is not rare since we have previously observed a reduction of CGA and other hormones at mRNA levels in response to other treatments in PitNETs [18]. Moreover, CgA has been detected in secretory granules of NFPT cells, its secretion has been employed as a useful marker of pharmacological effectiveness, and has been recently proposed as a biomarker of tumorigenesis and invasiveness since it was altered in invasive NFPTs [49,50,35], again supporting SST_3 as an attractive therapeutic target in NFPTs. In this sense, it is important to note that the development of the non-peptidic SST_3 agonist L-796,778 has been stopped and, therefore, the new peptides described in this work open a new avenue for the future development and use of SST_3-agonists to treat these tumors.

Interestingly, we found that a subset of NFPTs did not respond to SST_3-agonists in terms of inhibition of cell viability. This is not surprising, in that previous studies have shown a percentage of unresponsive NFPTs and functioning PitNETs, or even total absence of effect, upon treatment with different antitumor compounds [19,10,7]. A comparison of the expression profile of SSTs revealed higher SST_3 expression levels in responsive *vs.* unresponsive tumors, while no other significant changes were found between the two populations. Moreover, this result was corroborated at protein levels (higher SST_3 in responsive *vs.* unresponsive tumors), thus suggesting that high SST_3 levels are a requisite to elicit a significant response after SST_3-agonists administration. This notion is further supported by ROC-curve analysis demonstrating that only SST_3 levels, but not that of the other SST-subtypes, were able to discriminate between responsive and unresponsive NFPTs. SST_3-agonists were able to increase SST_3 expression levels in responsive, but not in unresponsive NFPTs (the latter showing even a reduction of SST_3 expression), thereby suggesting that a positive feedback, and thus a functionally unique SST_3(receptor)-*SSTR3*(gene) regulatory circuit might operate in these tumors. Remarkably, this is neither the sole nor the first time that an up-regulation of SSTs expression levels has been reported in response to different agonists. Thus, we have previously reported an increase of SST_2 and SST_5 expression levels in response to the chimeric compound BIM-23A760 in pituitary cells [18]. On the contrary, the reduction observed in unresponsive NFPTs could be due to an internalization/degradation of SST_3 in response to the agonist since it has been demonstrated in CHO-K1 cells overexpressing SST_3 that this receptor can undergo a rapid internalization (within minutes) in an agonist-dependent manner [17]. Nevertheless, the specific reasons why SST_3 reduction only occurs in unresponsive NFPTs need to be further explored.

To interrogate the mechanisms underlying the response to treatment with SST_3-agonists, we explored an ample range of signaling pathways. In contrast with the scarce association (if any) observed between $[Ca^{2+}]_i$ dynamics and response to SST_3-agonists, our data revealed, for the first time, a striking reduction on the

phosphorylation levels of relevant protein kinases associated to three important signaling pathways (i.e. MAPK, PI3K-AKT/mTOR and JAK/STAT). Specifically, we observed a decrease on ERK1/2 and JNK phosphorylation, as well as in other proteins of the MAPK pathway such as RSK1/2/3, CREB and c-Jun in response to BIM-355 in responsive NFPTs, but not in unresponsive NFPTs. In fact, these pathways have been commonly associated with cell growth, proliferation and cell survival in tumor pathologies, including NFPTs [39,46,37]. Moreover, our results demonstrating the importance of the MAPK pathway in the response to SST_3-agonists in NFPTs are in line with previous results on HEK-293 cells treated with L-796,778 [45]. Similarly, we also found a decreased phosphorylated status in Akt, GSK-3a/b, Src, PRAS40, mTOR and p70-S6 kinase, all of them associated to the PI3K/AKT/mTOR pathway, which controls cell cycle progression, protein synthesis and cell proliferation [37]. Indeed, it has been demonstrated that SSAs, such as octreotide, elicit their antiproliferative actions through inhibition of PI3K/AKT pathway [42], and also that specific inhibitors of mTOR pathway exhibit a potent antitumor efficacy in NFPTs [26]. Remarkably, all the changes in phosphorylation levels are consistent with reduced PI3K/AKT/mTOR signaling, except for p53, an important tumor suppressor, whose phosphorylation is involved in apoptosis. In fact, our results show a reduction on *TP53* mRNA expression and phosphorylated-p53 levels, which is in line with the reduction observed in C6 glioma cell in response to Acetaminophen [27]. It is well established that p53 is regulated by complex mechanisms. Nevertheless, the reduced levels of p53 remain to be confirmed. In any case, the caspase-mediated apoptotic effects observed in response to SST_3-agonists could be explained by the reduction in phosphorylated-CREB levels, which can reduce Bcl-2, an important anti-apoptotic protein, generating an increase on apoptosis [46]. Additionally, we observed a reduction on the phosphorylation levels of some components of JAK/STAT signaling pathway such as STAT3, STAT5a/b and STAT6. Although GPCRs have not been traditionally related with this signaling pathway, there are several reports describing an activation of STAT3 by GPCRs associated with cancer progression [48,24]. In marked contrast with responsive tumors, in unresponsive NFPTs, the phosphorylation status of most of the proteins described above were not altered upon BIM-355 treatment, or were increased. This situation is in accordance with results showing an increase on phosphorylation of Akt levels in a proportion of NFPTs resistant to rapamycin [5] and recent results published in prolactinomas showing that a reduced D2S/D2L expression ratio and an increase on MAPK and PI3K/AKT/mTOR pathways might contribute to tumorigenesis and DA resistance [38]. Moreover, comparing the basal phosphorylation levels of vehicle-treated controls from responsive and unresponsive NFPTs revealed a clear pattern of inhibition in the basal phosphorylation levels of the MAPK, PI3K-AKT/mT4OR and JAK/STAT proteins in unresponsive

tumors, which could explain the resistance of this population of NFPTs to respond to SST_3-agonists (and possibly to other SSAs).

Consistent with the existence of two populations of NFPTs (at least in terms of reduction of cell viability and signaling capacity in response to SST_3-agonists), the experimental silencing of *SSTR3* gene expression by specific siRNAs increased cell viability in 38% of NFPTs analyzed. A potential explanation for this observation would be that SST_3 bears a constitutive inhibitory activity, as previous results have demonstrated that various SSTs, including SST_3, display a relevant degree of ligand-independent constitutive activity in different pituitary cell systems [8,4]. Another possibility would be the endogenous release of somatostatin or its relative peptide cortistatin. In this sense, our results revealed almost negligible expression levels of somatostatin and much higher levels of cortistatin, which might suggest the existence of an ultra-short autocrine feedback mechanism whereby activation of SST_3 by endogenous cortistatin would mediate inhibition of cell viability, secretion, etc. Inversely, a reduction in SST_3 protein levels (by silencing) could lead to the loss of this inhibitory loop and could, ultimately, induce an increase on cell viability. However, the levels of somatostatin and cortistatin were not different between *SSTR3*-silencing responsive and unresponsive tumours and this hypothesis should be further explored. Importantly, the fact that BIM-355 did not alter cell viability in s13501-transfected cells (with reduced SST_3 content), together with the fact that SST_3-antagonist BIM-839 completely blocked the effects of SST_3-agonists, provide compelling evidence that, although cAMP IC50 for BIM-355 for SST_3 and SST_5 are very similar, the effects observed in response to agonists are completely and only specific of SST_3. These observations unveiled new conceptual and functional avenues in NFPTs, with potential therapeutic implications, which are worth exploring in future studies.

Taken together, this study represents the first identification and characterization of SST_3-peptidic agonists, which enabled to gain novel experimental results supporting that these agonists exert clear antitumor actions on NFPT primary cell cultures. Moreover, our data reveal a role of SST_3 in key pathophysiological processes of NFPT cells such as cell viability, apoptosis, and CgA secretion when SST_3 is targeted with specific agonists. These actions are likely mediated through the modulation of three important signaling pathways (MAPK, PI3K-AKT/mTOR and JAK/STAT). We also found two populations of NFPTs with a differential expression of SST_3 (statistically significant at the mRNA and protein level) and different capacity to respond to SST_3-agonists in terms of activation of signaling pathways and functional parameters. Therefore, the present study provides new compelling evidence, demonstrating that SST_3 has a functional role in the pathophysiology of NFPTs, and invites to suggest that pharmacological treatments specifically targeting this receptor could

become a promising option to treat patients with NFPTs, providing a relevant clinical conclusion, which should be soon tested for their use in humans.

Conflict of interest: M. Paez-Pereda is a current employee of IPSEN. G. Raverot, E. Venegas-Moreno, M.A. Gálvez, A. Soto-Moreno, J.P. Castaño and R.M. Luque have received lecture fees from Ipsen and Novartis. The rest of the authors have nothing to disclose.

Authors contribution: Conception and design of research: M.C.V.-B., V.G., A.I.C-C., M.P.P., J.P.C., R.M.L.; Performed experiments: M.C.V.-B., V.G., J.H., S.Z., H.H.; Analyzed data: M.C.V.-B, J.P.C., R.M.L.; Interpreted results: M.C.V.-B, A.I.C-C., M.D.G., M.P.P., M.D.C., J.P.C., R.M.L.; Prepared figures: M.C.V.-B., M.D.G., M.P.P., J.P.C., R.M.L.; Acquisition of clinical/pathological data and samples: E.V.-M., A.T.-D.,C.B.-A., J.S., R.O.-S., M.A.J., A.B.-M, A.V., M.A.-G., A.S.; Wrote the manuscript: M.C.V.-B., V.G., M.D.G., M.P.P., J.P.C., R.M.L.; Critically revised the manuscript and approved final version: M.C.V.-B., V.G., A.I.C-C., M.D.G., E.V.-M., A.T.-D., D.A.C, C.B.-A., R.O.-S., M.A.J., A.B.-M, A.V., J.H., S.Z., H.H., J.S., G.R., M.A.-G., A.S., M.P.P., M.D.C., J.P.C., R.M.L.

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FIGURE CAPTIONS

Figure 1. Chemical structures of selected SST_3-agonists and antagonist compounds. These compounds are representative of the families of peptides that showed the highest activity and selectivity for SST_3.

Figure 2. Functional assays in response to SST_3-agonists and antagonists in NFPTs primary cell cultures. (A) Effect of three agonists [10^{-7} M: BIM-355 (n=16), BIM-071 (n=13) and L-796,778 (n=18) on cell viability in responsive tumors (24-72 h treatment), measured by Alamar-blue reduction. (B) Effect of BIM-355 (n=4), BIM-071 (n=3) and L-796,778 (n=9) alone or in combination with antagonists (BIM-839 and BIM-152) on cell viability. (C) Effect of agonists (BIM-355 [n=5], BIM-071 and L-796,778 [n=3]) on caspase activity (24h treatment), measured by Caspase-Glo 3/7 assay. (D) Effect of agonists (BIM-355 [n=4], BIM-071 [n=3] and L-796,778 [n=8]) on chromogranin A secretion (24h treatment), determined by commercial ELISA kit. (E) mRNA expression levels of key genes in response to SST_3-agonists were measured by qPCR and adjusted by normalization factor (NF) (n=7). Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) indicate statistically significant differences.

Figure 3. Differential expression profile of key genes according to responsive or unresponsive NFPTs in terms of cell viability. (A) Expression profile of somatostatin receptors in responsive (RP, black bars; n=26) compared with unresponsive tumors (URP, grey bars; n=16) and ROC-curve analyses to determine the accuracy of SST_3 expression as diagnostic test to discriminate between RP and URP NFPTs. Data represent median ± interquartile range of absolute expression levels (copy number) of each transcript adjusted by the expression level of a control gene (*HPRT*). (B) SST_3 protein levels in both responsive (RP; n=17) and unresponsive (URP; n=12) NFPTs evaluated by IHC and ROC-curve analyses of SST_3 expression. Data represent median ± interquartile range. Asterisk (* $p<0.05$) indicate statistically significant differences. (C) Representative immunohistochemical staining (400X magnification; scale bar: 20μm) for SST_3 measured in RP and URP NFPTs. (D) Expression profile of SST_3 in both RP and URP tumors in response to treatment with SST_3-agonists (10^{-7} M) [RP (n=3) and URP (n=4): BIM-355, BIM-071 and L-796,778]. Data are expressed as percent of

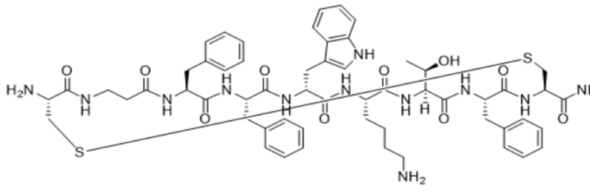
vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* $p<0.05$; ** $p<0.01$) indicate statistically significant differences.

Figure 4. Measurement of intracellular signaling pathways in response to BIM-355 in NFPTs. Direct effects of BIM-355 on the phosphorylation levels of 43 kinase phosphorylation sites, measured by phospho-kinase array. Array images are shown for responsive and unresponsive tumors treated with BIM-355 or vehicle. Graphs represent spot intensities of indicated proteins by quantifying the mean spot pixel densities. Values represent the mean ± SEM of duplicate spots from a pool of three NFPT cultures. Asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) indicate statistically significant differences.

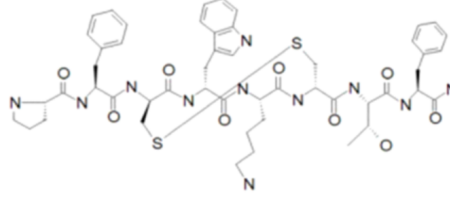
Figure 5. Cell viability in response to *SSTR3* gene silencing in NFPTs primary cell cultures. (A) Validation by qPCR of *SSTR3* gene silencing with two specific siRNAs (s13501 [n=9] and s224690 [n=6]). (B) Effect of 24-, 48- and 72-h silencing of SST_3 expression levels (non-responsive (n=5) and responsive (n=4) to silencing) on cell viability, determined by Alamar-blue reduction. (C) Basal SST_3 expression levels in responsive (RP, n=3) compared with non-responsive tumors (URP, n=5), before to perform the *SSTR3* gene silencing. (D) SST_3 expression levels in responsive (RP, n=3) compared with non-responsive tumors (URP, n=5). Data are expressed as percent of control random siRNA (Scramble; set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) indicate data that significantly differ from scramble controls.

Figure 6. Direct effect of BIM-355 on tumor growth in a preclinical PA mouse model. (A) Representative IHC of SST_3 in tumors generated in this model compared with SST_3 protein expression on human NFPTs (200X magnification; scale bar: 20µm). (B) Representative IHC of Ki67 in normal adjacent pituitary gland and tumors formed in this model (200X magnification; scale bar: 20µm). (C) Effect of BIM-355 [2.2 mg/kg/day (n=8) and 7.4 mg/kg/day (n=3)] on tumor volume growth during 8 weeks, measured by MRI. The asterisks represent statistical significance evaluated time-by-time by geometric least square values adjusted for baseline tumor volume. (D) Final tumor volume after 8 weeks of treatment. The bars represent geometric least square mean values corresponding to the complete curves from baseline to week 8, as described in Statistical Methods.

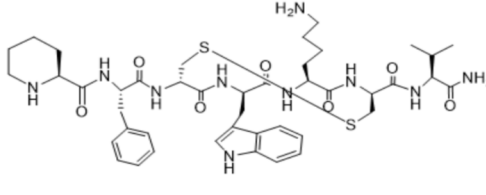
BIM-355: c(Cys-bAla-Phe-Phe-DTrp-Lys-Thr-Phe-Cys)-NH2

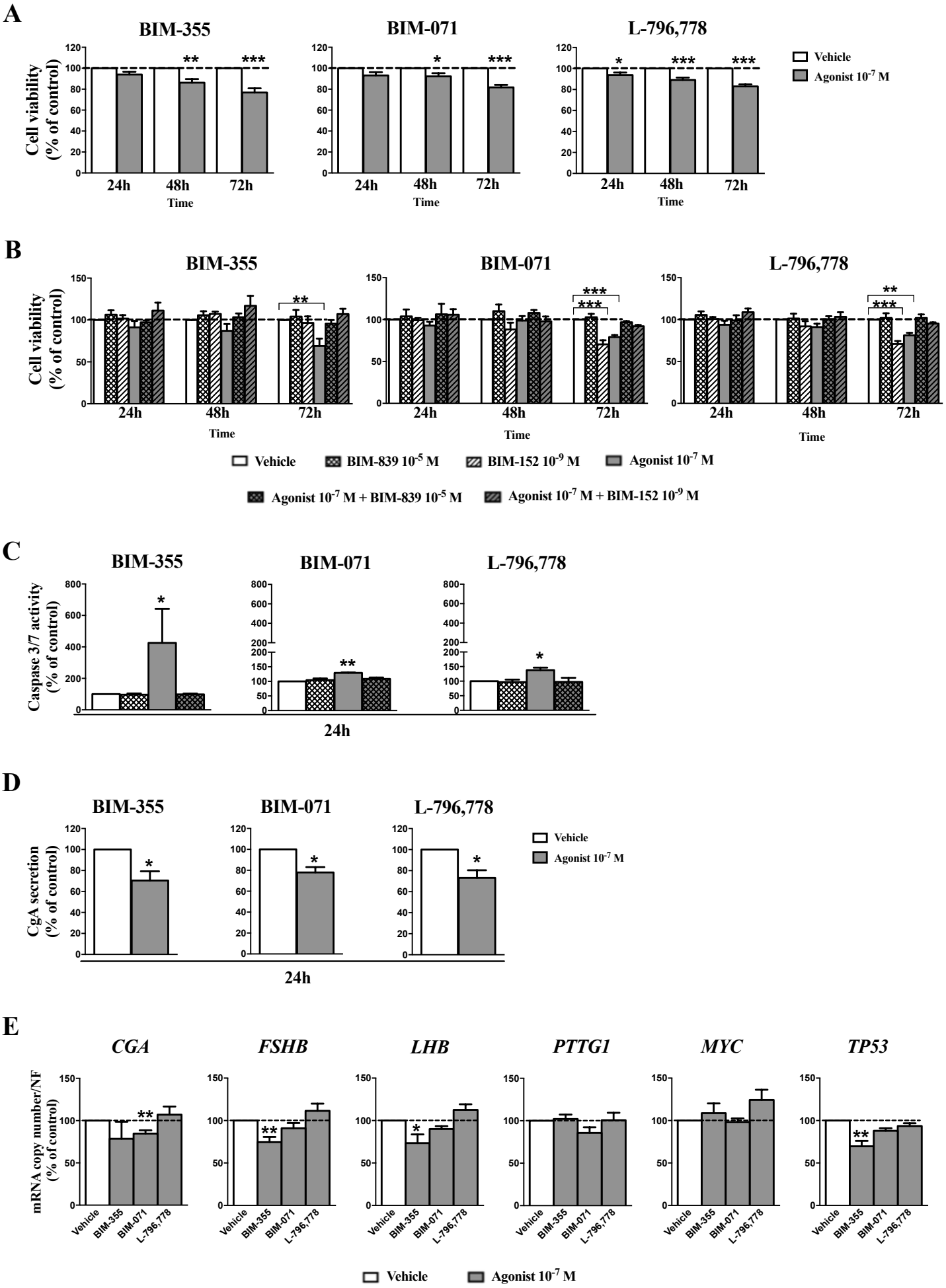


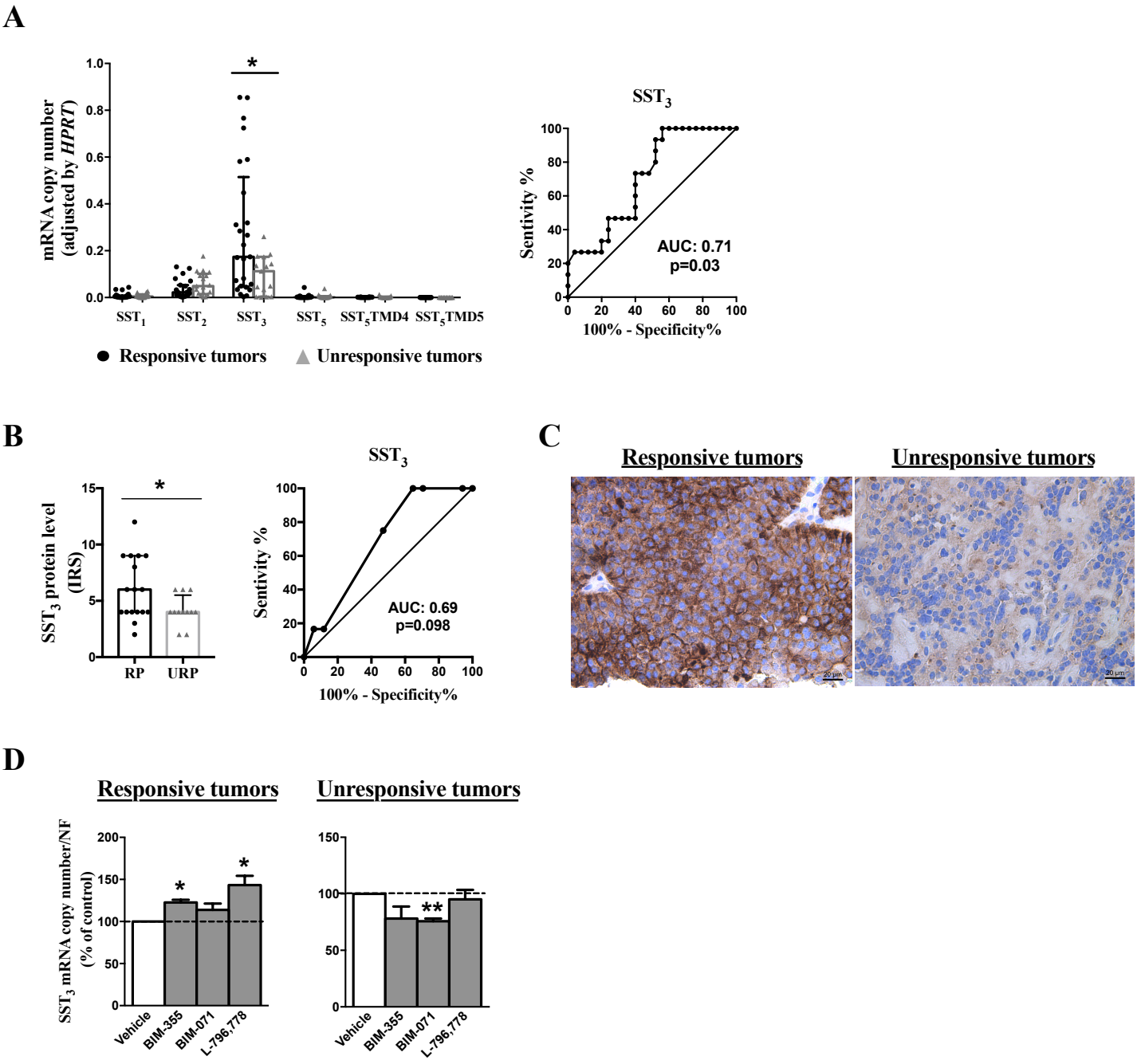
BIM-071: Pro-Phe-c(DCys-DTrp-Lys-DCys)-Thr-Phe-NH2

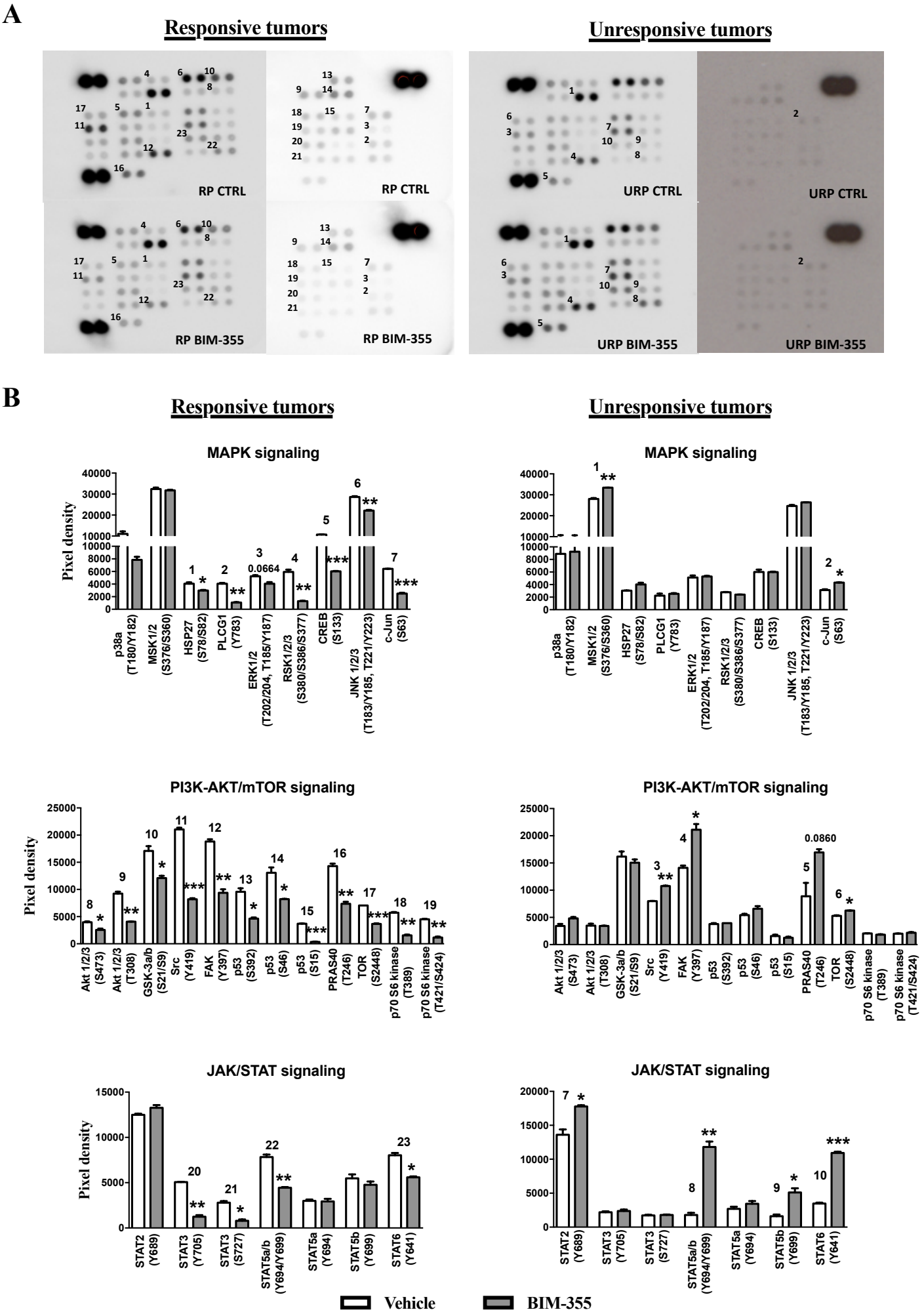


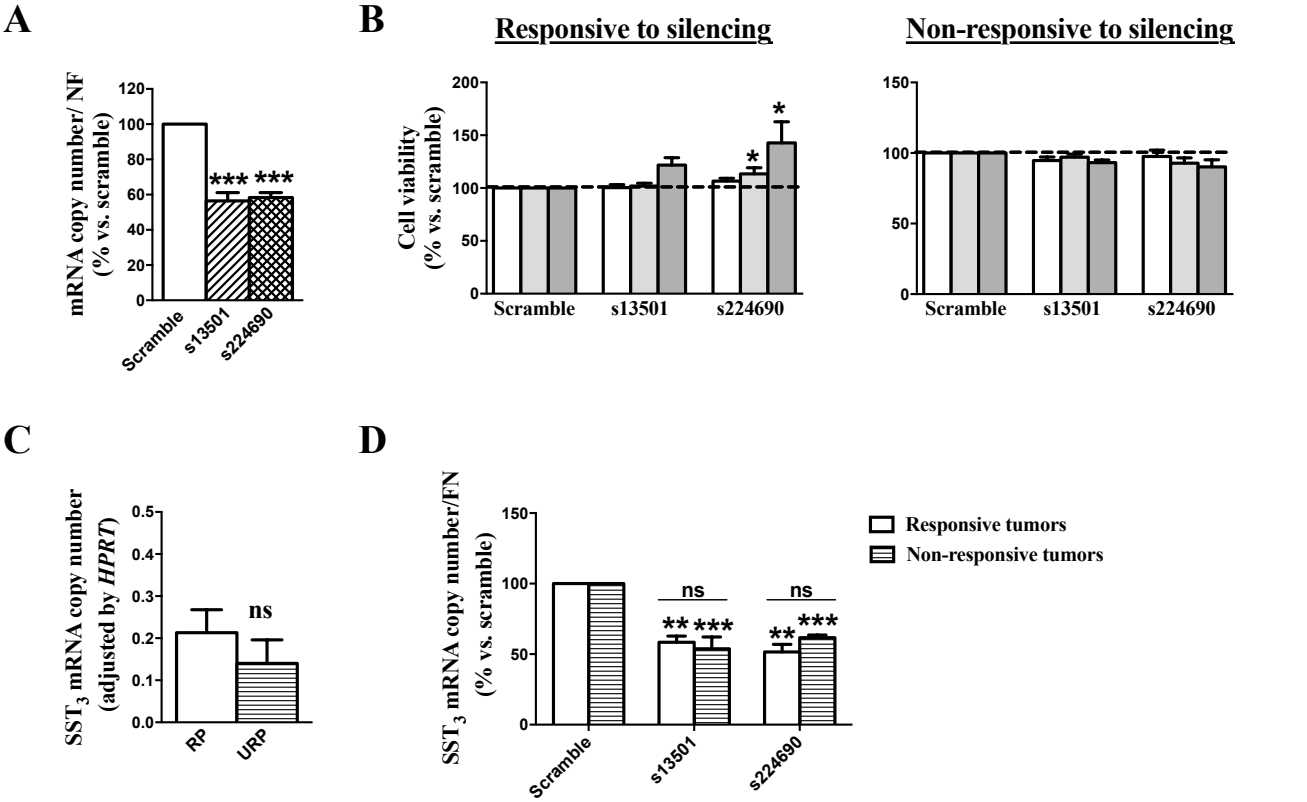
BIM-839: hPro-Phe-c(DCys-DTrp-Lys-DCys)-Val-NH2



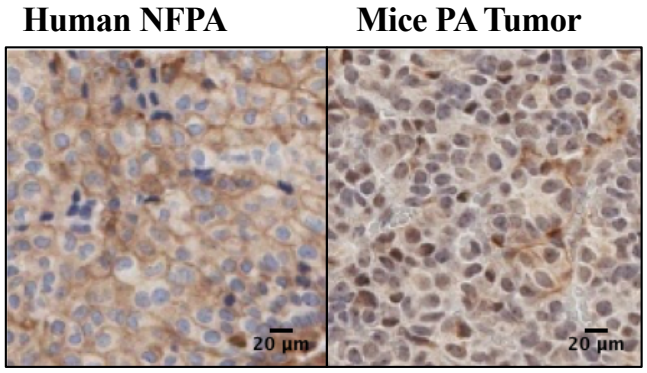

A
BIM-355
BIM-071
L-796,778
Cell viability (% of control)
Time
24h
48h
72h
Vehicle
Agonist 10-7 M
B
BIM-839 10-5 M
BIM-152 10-9 M
Agonist 10-7 M + BIM-839 10-5 M
Agonist 10-7 M + BIM-152 10-9 M
C
Caspase 3/7 activity (% of control)
D
CgA secretion (% of control)
E
CGA
FSHB
LHB
PTTG1
MYC
TP53
mRNA copy number/NF (% of control)
Vehicle
BIM-355
BIM-071
L-796,778


A
mRNA copy number (adjusted by HPRT)
1.0
0.8
0.6
0.4
0.2
0.0
*
SST1
SST2
SST3
SST5
SST5TMD4
SST5TMD5
Responsive tumors
Unresponsive tumors
SST3
Sentivity %
100
80
60
40
20
0
AUC: 0.71
p=0.03
0
20
40
60
80
100
100% - Specificity%
B
SST3 protein level (IRS)
15
10
5
0
*
RP
URP
SST3
Sentivity %
AUC: 0.69
p=0.098
100% - Specificity%
C
Responsive tumors
Unresponsive tumors
20 µm
20 µm
D
Responsive tumors
Unresponsive tumors
SST3 mRNA copy number/NF (% of control)
200
150
100
50
0
*
*
**
Vehicle
BIM-355
BIM-071
L-796,778

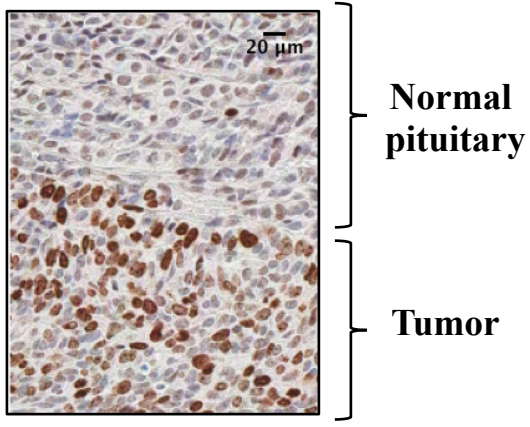

A
Responsive tumors
Unresponsive tumors
RP CTRL
RP BIM-355
URP CTRL
URP BIM-355
B
Responsive tumors
Unresponsive tumors
MAPK signaling
Pixel density
p38a (T180/Y182)
MSK1/2 (S376/S360)
HSP27 (S78/S82)
PLCG1 (Y783)
ERK1/2 (T202/204, T185/Y187)
RSK1/2/3 (S380/S386/S377)
CREB (S133)
JNK 1/2/3 (T183/Y185, T221/Y223)
c-Jun (S63)
PI3K-AKT/mTOR signaling
Akt 1/2/3 (S473)
Akt 1/2/3 (T308)
GSK-3a/b (S21/S9)
Src (Y419)
FAK (Y397)
p53 (S392)
p53 (S46)
p53 (S15)
PRAS40 (T246)
TOR (S2448)
p70 S6 kinase (T389)
p70 S6 kinase (T421/S424)
JAK/STAT signaling
STAT2 (Y689)
STAT3 (Y705)
STAT3 (S727)
STAT5a/b (Y694/Y699)
STAT5a (Y694)
STAT5b (Y699)
STAT6 (Y641)
Vehicle
BIM-355


A
mRNA copy number/ NF
(% vs. scramble)
120
100
80
60
40
20
0
*** ***
Scramble
s13501
s224690
B
Responsive to silencing
Non-responsive to silencing
Cell viability
(% vs. scramble)
200
150
100
50
0
* *
Scramble
s13501
s224690
150
100
50
0
Scramble
s13501
s224690
C
SST3 mRNA copy number
(adjusted by HPRT)
0.5
0.4
0.3
0.2
0.1
0.0
ns
RP
URP
D
SST3 mRNA copy number/FN
(% vs. scramble)
150
100
50
0
ns
ns
** ***
** ***
Scramble
s13501
s224690
Responsive tumors
Non-responsive tumors

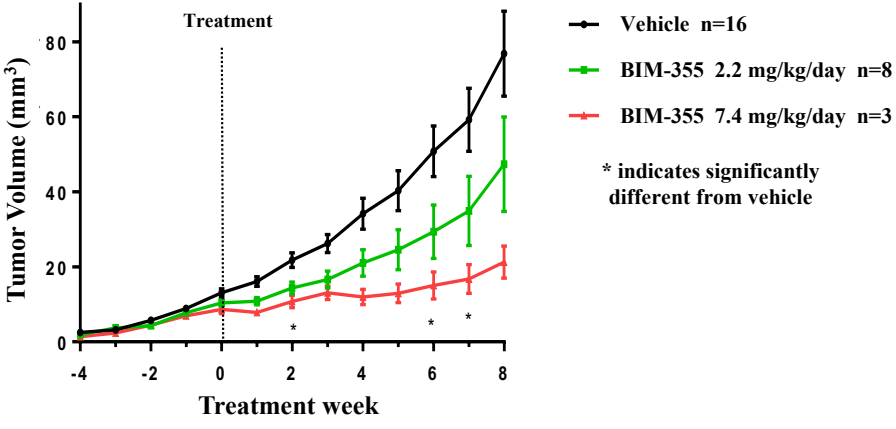
A


Human NFPA
Mice PA Tumor
20 µm
20 µm

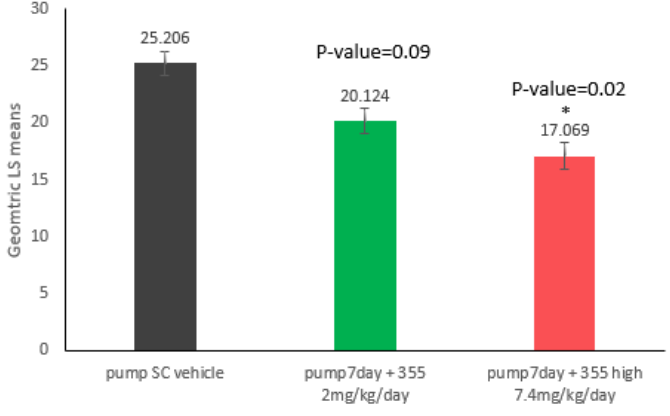
B


20 µm
Normal pituitary
Tumor

C


Treatment
Tumor Volume (mm3)
Treatment week
Vehicle n=16
BIM-355 2.2 mg/kg/day n=8
BIM-355 7.4 mg/kg/day n=3
* indicates significantly different from vehicle

D


Geomtric LS means
25.206
P-value=0.09
20.124
P-value=0.02
*
17.069
pump SC vehicle
pump7day + 355 2mg/kg/day
pump7day + 355 high 7.4mg/kg/day

Supplementary Information

A novel SST_3 agonist shows potential antitumor effects in experimental models of Nonfunctioning Pituitary Tumors

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Methods

Library screening

The identification of compounds that act as selective SST_3-agonists was based on the examination of IPSEN's extensive collection of synthetic peptides. First, we measured the affinity of all the peptides to SST_2, SST_3, and SST_5 as previously described [1]. Then, peptides that showed binding to SST_3 regardless of their potency were included in a multidimensional Spotfire® analysis designed to elucidate compounds with the highest selectivity for SST_3. Particular focus was paid to compounds that had simultaneous selectivity against SST_2 and SST_5. Structural analysis of the most selective compounds allowed them to be classified into 3 main families with 6, 4 and 2 members respectively. The most representative structures are BIM-355, BIM-071 and BIM-23A185. High throughput synthesis of family members from each group allowed the confirmation of their SST_3 binding, selectivity, and cellular activity. The activation of SSTs was performed by measuring cAMP as previously described [1].

Patients, samples and primary cell cultures

The appropriate classification of each pituitary sample collected (NP or tumor type) was confirmed by two different methods: examination by expert anatomo-pathologists and by the molecular screening using quantitative real-time PCR (qPCR) as previously described [2-4].

For *in vitro* assays, fresh tumor samples were placed in sterile cold media and dispersed into single cells by mechanical and enzymatic disruption and cultured onto different tissue culture plates following the methods and reagents previously reported [2,4].

RNA isolation, reverse transcription and analysis of gene expression levels by qPCR

We have evaluated the stability of the expression of three reference genes *ACTB*, *HPRT* and *GAPDH* in all samples using RefFinder, a comprehensive tool that integrates the currently available major computational programmes [5], and found *HPRT* to be the most stable. Taking this into account, the expression values of the genes of interest were normalized to *HPRT* mRNA levels.

Immunohistochemical analysis (IHC) for SST_3 in NFPTs

The presence of antigen was revealed with the UltraView Universal DAB Detection Kit (Ventana). SST_3 antibody was diluted 1/1000 (UMB-5, Abcam, Burlingame, CA). An immunoreactive

score (IRS) was established for each section. This score was calculated (ranging from 0 to 12) as the product of the percentage of positive cells (0-4: 0, ≤10, 11-50, 51-79 and ≥80%, respectively) and the staining intensity (0-3: no staining, mild, moderate and strong, respectively). The slides were semi-quantitatively scored by two independent, experienced pathologists by a double-blind method and similar results were obtained.

Measurement of caspase activity of primary pituitary tumor cell cultures

For this purpose, 25,000 cells/well were plated in a 96-well white microplate and cultured for 24h at 37°C in an atmosphere containing 5% CO_2. Then, cells were incubated for another 24h with different SST_3-agonists, BIM-839 antagonist and vehicle. After the incubation period, 100µl of Caspase-Glo 3/7 reagent was added to each well and luminescence was measured at room temperature using FlexStation III system for 3h.

Analysis of signaling pathways by human phospho-kinase array.

Three different NFPTs of each condition (vehicle/treatment; responsive/unresponsive NFPTs) were pooled and 600µg of cell lysates were loaded in the array membranes to detect relative phosphorylation levels of 43 relevant kinase phosphorylation sites, following the manufacturer´s protocol (Proteome Profiler™; R&D Systems). The average signal of the pair of duplicate spots, representing each phosphorylated site, was calculated after subtraction of background values (pixel density) from negative control spots and normalization to average values from positive control spots using HLImage++ sofware (Version 22.0.0a; R&D Systems).

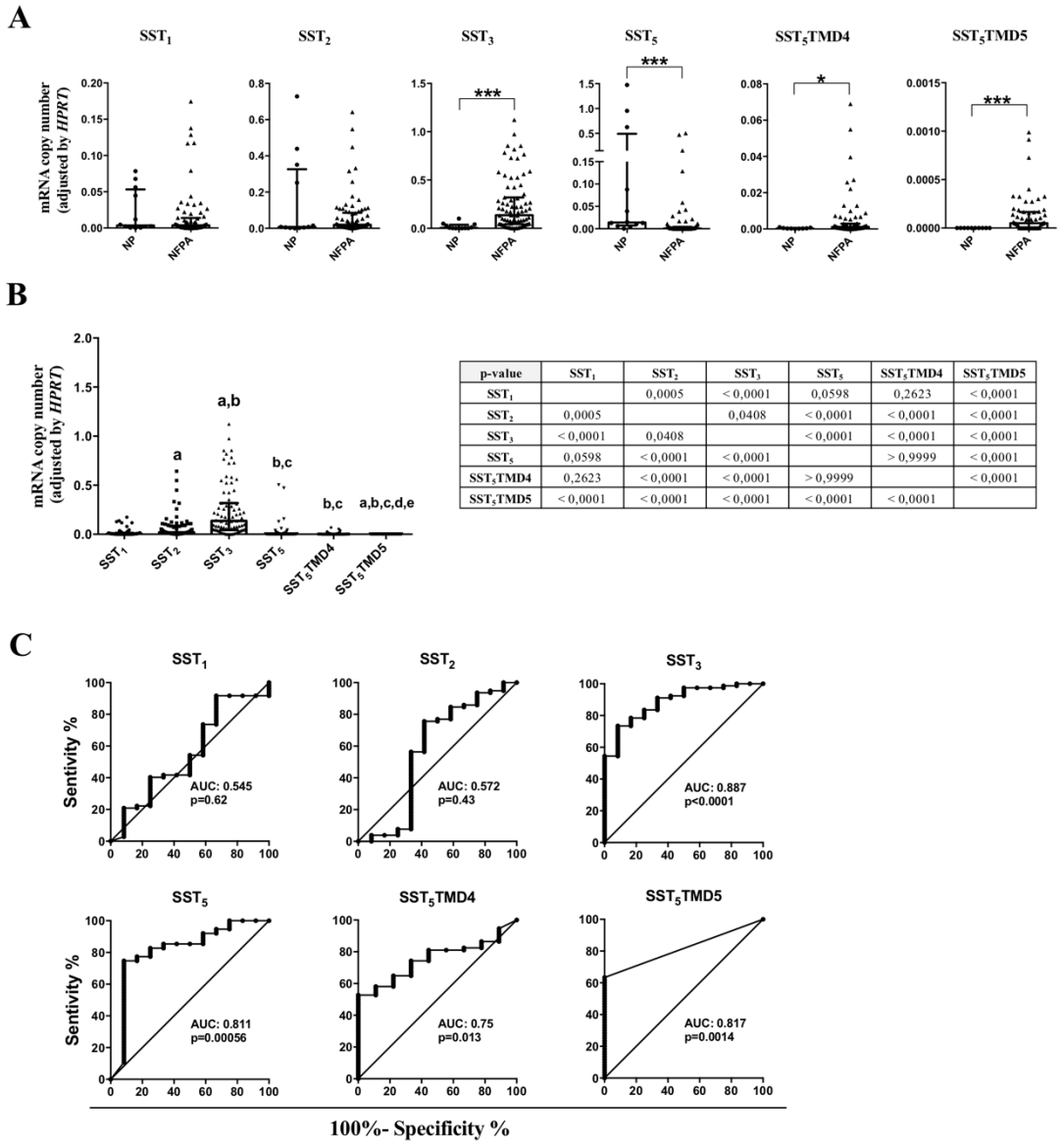
Silencing of SSTR3 gene with specific siRNAs

The validation of silencing conditions was carried out using the BON-1 cell line, which displays a high SST_3 expression (data not shown). After 24h of transfection, cells were detached and used for validation of the transfection efficiency (determined by qPCR) and for cell viability measurements as described above.

Validation of SST_3 silencing by western blotting.

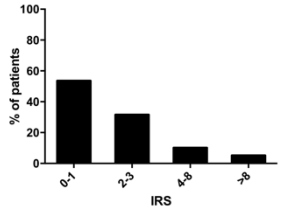
To assess the SST_3 protein reduction in response to the silencing, 300,000-500,000 transfected cells (scramble and s13501) were washed and lysed in SDS-DTT buffer after 48h of transfection. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0,05% Tween 20 and incubated overnight a 4ºC with primary antibodies [anti-SST_3: UMB5, ab137026 (Abcam, Cambridge, UK); anti-β-tubulin: 2128S (Cell Signaling, Danvers, MA, USA)]. Secondary horseradish peroxidase-conjugated anti-rabbit were purchased from Cell Signaling (Danvers, MA, USA). Proteins were developed by using ECL detection system (GE Healthcare, UK) with dyed molecular weight markers. A densitometric analysis of the bands was carried out with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Fig S1. mRNA and protein expression pattern of key somatostatin receptors in non-functioning pituitary tumors (NFPTs) and normal pituitaries (NPs). (A) The expression profile of somatostatin receptors was determined by qPCR in a battery of 80 NFPTs and 12 NPs. (B) Comparative expression profile of somatostatin system in 80 NFPTs. Data represent median ± interquartile range of absolute expression levels (copy number) of each transcript adjusted by the expression level of a control gene (*HPRT*). Asterisks (* p<0.05; ** p<0.01; *** p<0.001) and values that do not share a common letter indicate statistically significant differences. (C) ROC-curve analyses to determine the accuracy of SSTs expression levels to discriminate between patients with NFPTs and normal tissue (NPs). (D) Immunohistochemical staining (400X magnification; scale bar: 20μm) and immunoreactive score (IRS) for SST_3 measured in two different cohorts of NFPTs (n=35).



p-value	SST_1	SST_2	SST_3	SST_5	SST_5TMD4	SST_5TMD5
SST_1		0,0005	< 0,0001	0,0598	0,2623	< 0,0001
SST_2	0,0005		0,0408	< 0,0001	< 0,0001	< 0,0001
SST_3	< 0,0001	0,0408		< 0,0001	< 0,0001	< 0,0001
SST_5	0,0598	< 0,0001	< 0,0001		> 0,9999	< 0,0001
SST_5TMD4	0,2623	< 0,0001	< 0,0001	> 0,9999		< 0,0001
SST_5TMD5	< 0,0001	< 0,0001	< 0,0001	< 0,0001	< 0,0001	

D


100
80
60
40
20
0
% of patients
0-1
2-3
4-8
>8
IRS

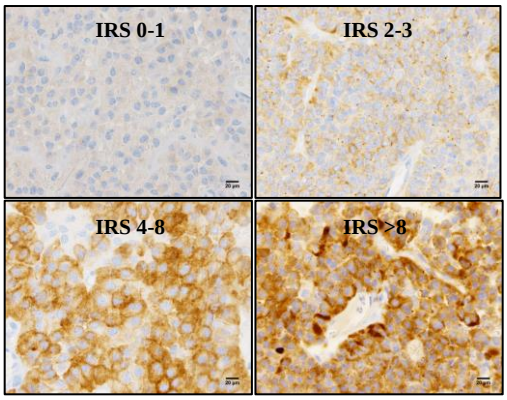

IRS 0-1
IRS 2-3
IRS 4-8
IRS >8

Fig. S2. mRNA expression levels of SST_3 in different pituitary neuroendocrine tumors (PitNETs) and NPs. (A) Comparative expression profile of SST_3 between NFPTs (n=80), functioning PitNETs [GHomas (n=63) and ACTHomas (n=17)] and NPs (n=12). (B-D) ROC-curve analyses to determine the accuracy of SST_3 expression to discriminate between (B) patients with NFPTs and functioning PitNETs, (C) between NPs and GHomas or ACTHomas and (D) between GHomas or ACTHomas. (E) Association between SST_3 mRNA expression levels and LHB mRNA expression and clinical parameters. Data represent median ± interquartile range of absolute expression levels (copy number) of each transcript adjusted by the expression level of a control gene (*HPRT*). Values that do not share a common letter indicate statistically significant differences.

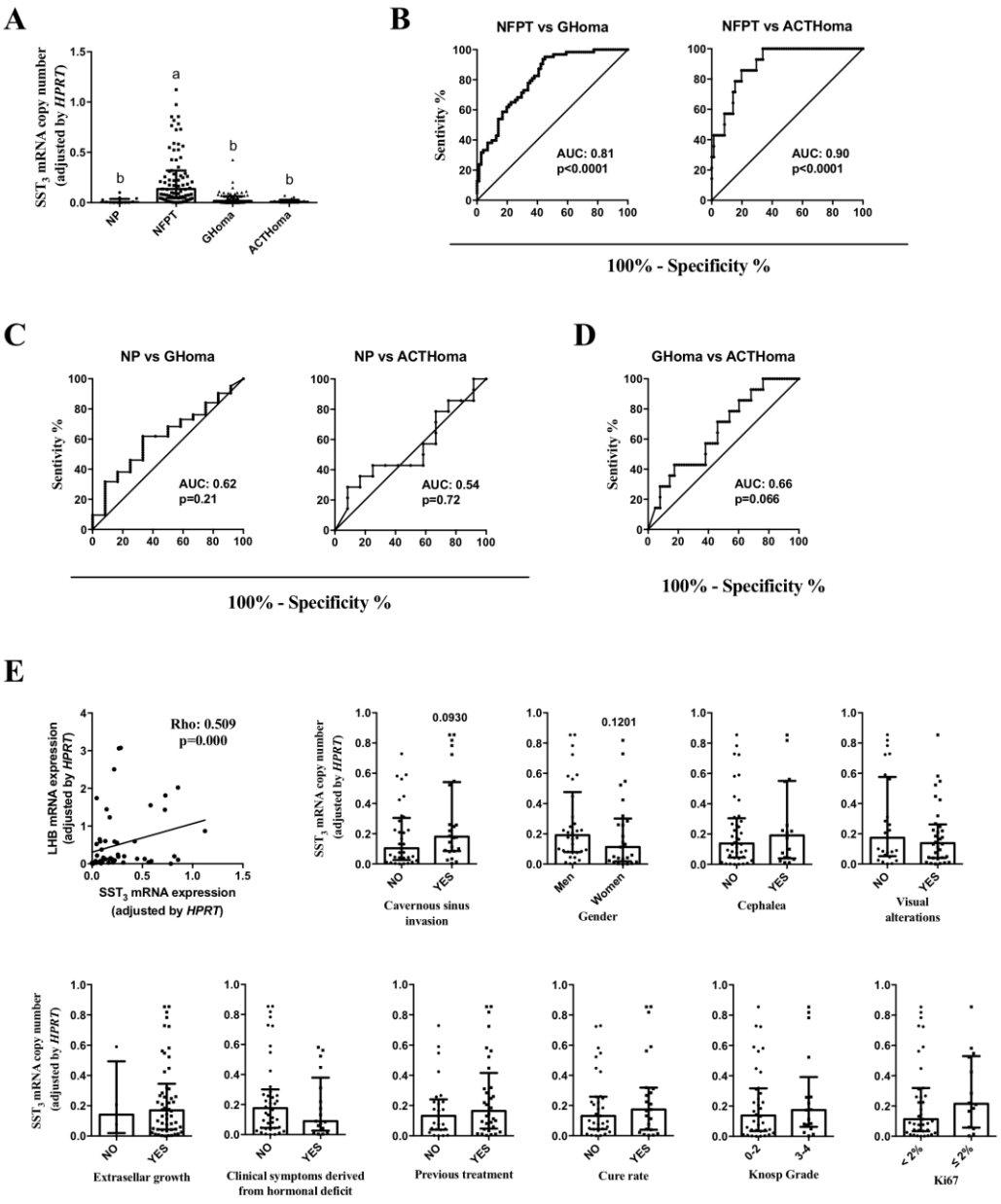


Fig. S3. Cell viability in response to SST_3-agonists and antagonists in NFPTs primary cell cultures, measured by Alamar-blue reduction. (A) Concentration-dependent response experiments of SST_3-agonists [BIM-355 (n=4), BIM-071 (n=4), and L-796,778 (n=5)]. (B) Concentration-dependent response experiments of two SST_3-antagonists (BIM-839 [n=3] and BIM-152 [n=9]). Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (** $p<0.01$; *** $p<0.001$) indicate statistically significant differences.

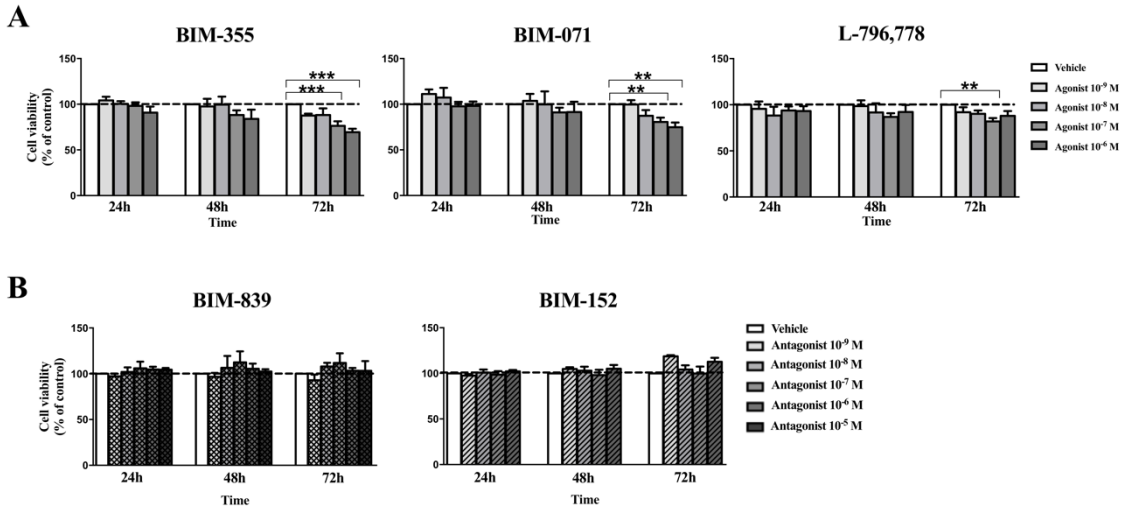


Fig. S4. ROC-curve analyses to determine the accuracy of SSTs expression levels to discriminate between responsive and unresponsive NFPTs.

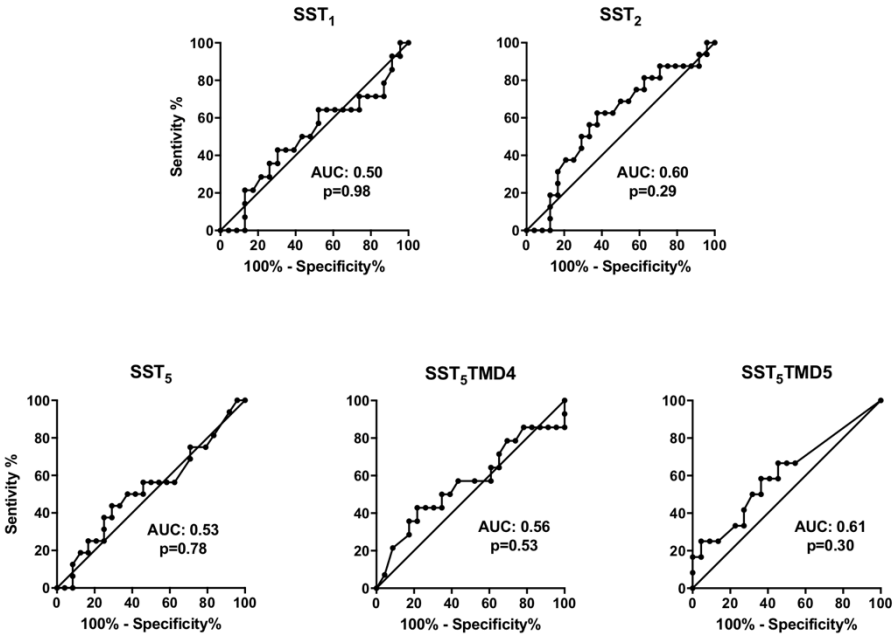
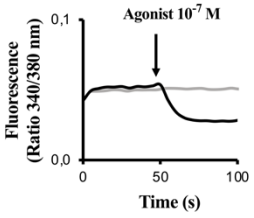


Fig. S5. Intracellular signaling involved in the effects observed in response to SST_3-agonists. (A) Summarized table and representative profile of $[Ca^{2+}]_i$ kinetics assay in response to different agonists (BIM-355 [n=4], BIM-071 and L-796,778 [n=11]). n stands for "responsive samples/total samples analyzed"; PRC, proportion of responsive cells showing changes in $[Ca^{2+}]_i$ in response to agonists; PMR, percentage of maximum response; and time, time of response to agonists administration. (B) Comparison of the basal phosphorylation levels of vehicle-treated controls from responsive and unresponsive NFPTs, measured by phospho-kinase array. Graphs represent spot intensities of indicated proteins by quantifying the mean spot pixel densities. Values represent the mean ± SEM of duplicate spots from a pool of three NFPT cultures. Asterisks (* p <0.05; ** p <0.01; *** p <0.001) indicate statistically significant differences.

A

Treatment	% PRC	% PMR ± SEM	Time (s) ± SEM
BIM-355	12,4	74,04 ± 2,50	50,3 ± 6,39
BIM-071	10	73,3 ± 3,6	70 ± 6,79
L-796,778	7	72,64 ± 2,45	46,13 ± 2,74



B

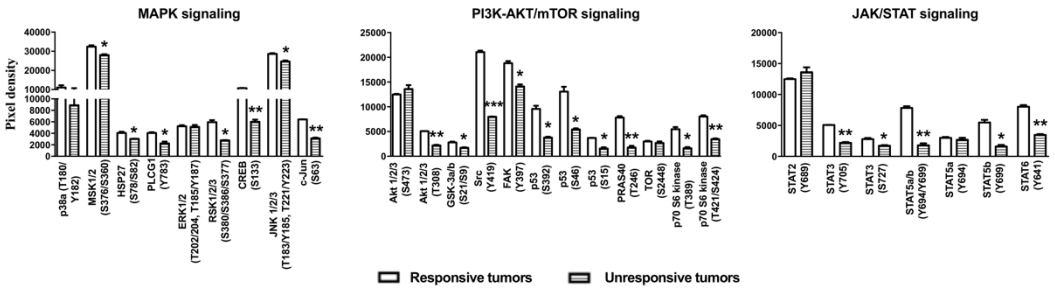


Fig. S6. Cell viability in response to BIM-355 and *SSTR3* gene silencing in NFPT primary cell cultures. (A) Validation by western blotting of SST_3 silencing after 48h of transfection using s13501 (n=1). (B) Validation by western blotting of SST_3 silencing after 72h of transfection using s13501 and s224690 siRNA (n=1). (C) Effect of 24-, 48- and 72-h silencing of SST_3 protein alone or in combination with BIM-355 treatment on cell viability (n=1), determined by Alamar-blue reduction. Data are expressed as percent of vehicle-treated controls (set at 100%). Values represent the mean ± SEM. Asterisks (** $p<0.01$) indicate statistically significant differences between a given treatment vs. vehicle-treated control using the replicates (n=4) within each treatment.

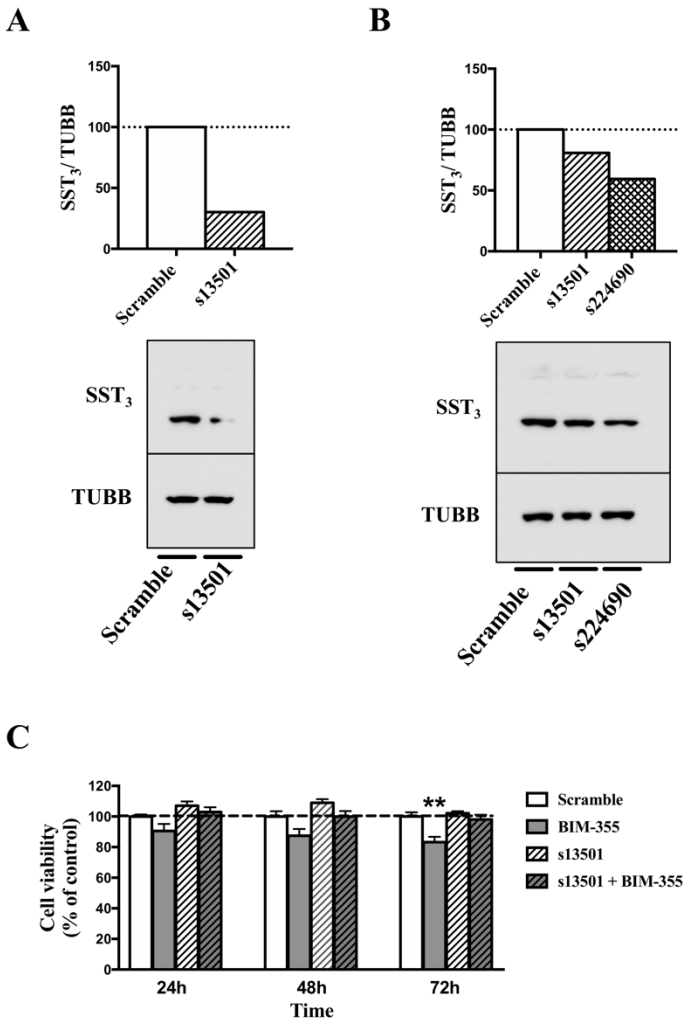


Fig. S7. Direct effect of BIM-355 at different doses on tumor growth in POMC-KO mice model.

Graphs are representing the tumor volume measured by MRI in individual mice.

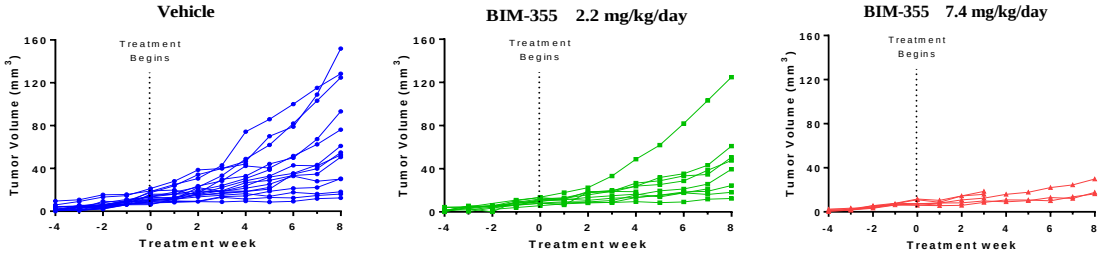


Table S1. Pharmacological characterization of SST_3-selective compounds

Compound	Type	Molecular weight (g/mol)	Ki (nM)	cAMP assay IC50 (nM)				
			$hSST_3$	$hSST_2$	$hSST_3$	$hSST_5$	$mSST_3$	$mSST_5$
BIM-355	Peptidic agonist	1269,5	1.98 ± 0.1	224 ± 73	4 ± 0.8	5.7 ± 1	19 ± 6.3	229 ± 36
BIM-071	Peptidic agonist	1028,3	3.55 ± 0.5	62.7 ± 12.8	1.8 ± 0.5	>1000	>1000	ND
L-796,778	Non-peptidic agonist	585,7	ND	ND	18 [8]	ND	ND	ND
BIM-839	Peptidic antagonist	1121,2	11.92 ± 7.0	>1000	2 ± 0.5	>500	ND	ND

ND: Not determined

Table S2. Relation of functional experiments performed in responsive (RP) and unresponsive (URP) NFPTs.

Sample	Type of response	mRNA expression at basal levels (from tissue)	IHC	Cell viability	Caspase 3/7 activity	Chromogranin A secretion	mRNA expression
NFPT-1	RP	X	X	X	-	-	-
NFPT-2	RP	X	X	X	-	X	X
NFPT-3	RP	X	X	X	-	X	X
NFPT-4	RP	X	X	X	-	-	-
NFPT-5	RP	X	X	X	-	-	-
NFPT-6	RP	X	X	X	-	X	-
NFPT-7	RP	X	X	X	-	-	-
NFPT-8	RP	X	X	X	-	-	-
NFPT-9	RP	X		X	-	X	X
NFPT-10	RP	X	X	X	-	-	-
NFPT-11	RP	X		X	-	X	X
NFPT-12	RP	X		X	-	X	X
NFPT-13	RP	X		X	-	-	-
NFPT-14	RP	X		X	-	-	-
NFPT-15	RP	X		X	-	-	-
NFPT-16	RP	X		X	-	-	-
NFPT-17	RP	X		X	-	-	-
NFPT-18	RP	X	X	X	-	-	-
NFPT-19	RP	X	X	X	X	X	X
NFPT-20	RP	X	X	X	X	-	-
NFPT-21	RP	X	X	X	X	-	-
NFPT-22	RP	X		X	X	X	X
NFPT-23	RP	X	X	X	X	-	-
NFPT-24	RP	X	X	X	-	-	-
NFPT-25	RP	X	X	X	-	-	-
NFPT-26	RP	X	X	X	-	-	-
NFPT-27	URP	X	X	X	-	-	-
NFPT-28	URP	X		X	-	-	-
NFPT-29	URP	X	X	X	-	-	-
NFPT-30	URP	X	X	X	-	X	X
NFPT-31	URP	X	X	X	-	X	X
NFPT-32	URP	X		X	-	X	X
NFPT-33	URP	X	X	X	-	-	X
NFPT-34	URP	X	X	X	-	-	-

NFPT-35	URP	X	X	X	-	-	-
NFPT-36	URP	X	X	X	-	-	-
NFPT-37	URP	X	X	X	-	-	-
NFPT-38	URP	X	X	X	-	-	-
NFPT-39	URP	X	X	X	-	-	-
NFPT-40	URP	X		X	-	-	-
NFPT-41	URP	X	X	X	-	-	-
NFPT-42	URP	X		X	-	-	-

Table S3. Percentage of responsive and unresponsive NFPTs regarding SST_3-specific agonists in terms of cell viability.

	BIM-355	**BIM-071**	**L-796,778**
Responsive tumors	70% (n=16)	59% (n=13)	55% (n=18)
Unresponsive tumors	30% (n=7)	41% (n=9)	45% (n=15)
Total number of samples	N=23	N=22	N=33

Table S4. Results from Chi-square test of clinical parameters between responsive and unresponsive NFPTs.

Clinical parameter	**Chi-squared test**	**p-value**
Cephalea	0.178	1.000
Visual alterations	0.009	1.000
Extrasellar growth	0.197	1.000
Cavernous sinus invasion	0,433	0.707
Chiasmatic compression	0.423	0.601
Clinical symptoms derived from hormonal deficit	0.134	1
Pre-surgery treatment	0.029	1.000
Knosp Grade	0.008	1.000
Ki67 (<2% or ≥2%)	0.117	1.000
Cure rate	3.463	0.135

Table S5. Clinical data and SST_3 mRNA expression levels of individual NFPTs.

Nº	Gender	Age	Cephalea	Visual Alterations	Extrasellar growth	Cavernous sinus invasion	Knosp Grade	Cure rate	Ki67 (%)
1	F	53	Yes	No	Yes	No	2	Yes	<2%
2	F	71	Yes	Yes	Yes	No	0	No	<2%
3	M	73	No	Yes	Yes	No	0	Yes	<2%
4	F	52	No	No	Yes	No	0	No	<2%
5	M	67	Yes	No	Yes	No	0	No	<2%
6	F	51	No	Yes	Yes	No	0	No	<2%
7	F	67	Yes	No	Yes	Yes	3	Yes	<2%
8	M	41	Yes	Yes	Yes	Yes	3	No	= or >2%
9	F	57	Yes	ND	Yes	Yes	4	No	<2%
10	M	73	Yes	Yes	Yes	No	0	No	<2%
11	M	54	No	No	Yes	Yes	3	ND	<2%
12	M	39	Yes	No	Yes	No	2	Yes	<2%
13	F	43	No	Yes	Yes	No	0	Yes	<2%
14	M	74	Yes	Yes	Yes	No	2	Yes	<2%
15	F	50	No	No	Yes	No	1	No	= or >2%
16	F	71	Yes	Yes	Yes	No	2	No	<2%
17	M	64	No	No	Yes	Yes	4	No	<2%
18	M	43	No	Yes	Yes	No	0	Yes	<2%
19	M	56	No	Yes	Yes	No	2	No	<2%
20	F	42	No	Yes	Yes	No	1	Yes	<2%
21	F	71	ND	Yes	Yes	Yes	3	No	= or >2%
22	F	57	No	Yes	Yes	No	0	No	= or >2%
23	M	51	No	Yes	Yes	No	2	Yes	<2%
24	M	55	No	No	No	No	2	Yes	<2%
25	M	44	Yes	No	Yes	Yes	3	Yes	ND
26	M	37	No	No	Yes	Yes	3	Yes	<2%
27	F	47	No	Yes	Yes	No	0	Yes	<2%
28	M	75	No	Yes	Yes	Yes	3	No	<2%
29	F	51	No	Yes	Yes	No	0	No	= or >2%
30	M	40	No	Yes	Yes	ND	ND	Yes	<2%
31	M	40	No	Yes	Yes	No	0	Yes	<2%
32	M	65	No	Yes	Yes	No	2	No	= or >2%
33	M	68	No	No	Yes	No	2	Yes	<2%
34	F	73	No	Yes	Yes	No	ND	No	ND
35	M	57	No	No	Yes	Yes	4	No	<2%
36	M	51	Yes	No	Yes	No	2	No	ND
37	M	58	No	No	No	No	1	No	ND
38	M	63	No	No	Yes	Yes	0	Yes	= or >2%
39	M	43	No	No	No	No	3	No	<2%
40	F	65	No	No	Yes	Yes	4	No	<2%
41	F	52	No	Yes	Yes	Yes	1	No	<2%
42	F	66	No	No	Yes	No	1	Yes	= or >2%
43	M	67	Yes	No	Yes	Yes	1	No	<2%
44	M	75	No	No	Yes	Yes	4	No	<2%
45	F	32	No	Yes	Yes	No	4	No	= or >2%
46	M	16	Yes	Yes	Yes	No	ND	Yes	= or >2%
47	M	44	Yes	Yes	Yes	No	1	No	<2%
48	M	58	No	Yes	ND	ND	ND	No	ND
49	F	48	Yes	Yes	Yes	Yes	ND	No	= or >2%
50	F	79	No	Yes	Yes	Yes	1	No	= or >2%
51	M	59	Yes	Yes	Yes	Yes	2	Yes	<2%
52	F	65	No	Yes	Yes	Yes	3	Yes	<2%

53	F	38	No	No	Yes	Yes	3	Yes	= or >2%
54	M	72	No	No	Yes	Yes	3	No	<2%
55	M	51	No	Yes	Yes	Yes	1	No	ND
56	F	73	Yes	Yes	Yes	Yes	1	No	<2%
57	F	41	No	No	No	No	1	No	<2%
58	M	48	No	Yes	Yes	No	2	Yes	= or >2%
59	M	74	No	No	Yes	Yes	2	ND	<2%

NA: Not available; ND: Not determined

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A new generation somatostatin-dopamine analogue exerts potent antitumoral actions on pituitary neuroendocrine tumor cells

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Short Title: Effects of BIM-065 on pituitary tumors.

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Keywords: pituitary neuroendocrine tumors, chimeric compound, somatostatin, dopamine, receptor.

1. Abstract

Background: Pituitary neuroendocrine tumors (PitNETs) represent approximately 15% of all intracranial tumors and usually are associated with severe comorbidities. Unfortunately, a relevant number of patients do not respond to currently available pharmacological treatments, i.e. somatostatin-analogues (SSAs) or dopamine-agonists (DA). Thus, novel, chimeric somatostatin/dopamine compounds (dopastatins) that could improve medical treatment of PitNETs have been designed.

Objective: This study aims to determine the direct therapeutic effects of a new-generation dopastatin, BIM-065, on primary cell cultures from different PitNETs subtypes.

Methods: 31 PitNET-derived cell-cultures [9 corticotropinomas, 9 somatotropinomas, 11 non-functioning pituitary adenomas (NFPAs) and 2 prolactinomas], were treated with BIM-065, and key functional endpoints were assessed (cell viability, apoptosis, hormone secretion, expression levels of key genes, free cytosolic $[Ca^{2+}]_i$ dynamics, etc.). AtT-20 cell line was used to evaluate signaling pathways in response to BIM-065.

Results: This chimeric compound decreased cell viability in all corticotropinomas and somatotropinomas tested, but not in NFPAs. BIM-065 reduced ACTH, GH, chromogranin-A and PRL secretion, and increased apoptosis in corticotropinomas, somatotropinomas and NFPAs. These effects were possibly mediated through modulation of pivotal signaling cascades like $[Ca^{2+}]_i$ mobilization and Akt- or ERK1/2-phosphorylation.

Conclusions: Our results unveil a robust antitumoral effect *in vitro* of the novel chimeric compound BIM-065 on the main PitNET-subtypes, inform on the mechanisms involved, and suggest that BIM-065 could be an efficacious therapeutic option to be considered in the treatment of PitNETs.

2. Introduction

Pituitary neuroendocrine tumors (PitNETs) are mostly benign neuroendocrine neoplasms confined to the *sella turcica* that constitute approximately 15% of all intracranial tumors [1, 2]. These tumors are usually monoclonal, derived from the expansion of single precursor cells, and are associated with severe comorbidities related to hormonal hypersecretion and/or mass effects, such as hypopituitarism, visual defects, amenorrhea, galactorrhea, sexual dysfunctions and growth abnormalities [3-5]. The expression of somatostatin and dopamine receptors (SST_{1-5} and D_{1-5}, respectively) has been largely studied in the different PitNETs subtypes, wherein high levels of certain SSTs/Ds, especially SST_2, SST_5 and D_2, have been described [6-10]. For this reason, somatostatin analogues (SSAs: i.e. octreotide, pasireotide and lanreotide) and dopamine agonists (i.e. cabergoline and bromocriptine) have been widely used as pharmacological treatments to manage this and other endocrine-related pathologies due to their multiple antiproliferative and antisecretory actions [6, 11, 12]. Nevertheless, numerous reports have demonstrated that a high proportion of patients are unable to respond to these treatments, or that the responses show a high variability or decline over time [12-14]. Inasmuch as PitNETs often express, simultaneously, high levels of various SSTs and Ds, and both families of receptors have the capacity of heterodimerize resulting in altered pharmacological properties [15, 16], new chimeric somatostatin (SRIF)/ Dopamine (DA) compounds were developed in order to increase their efficacy and improve the control of the disease compared with individual SSA and/or DA [17].

In this context, BIM-23A760, a chimeric compound able to bind SST_2, SST_5 and D_2 with high affinity, was considered a promising therapeutic option to treat different PitNETs subtypes. In fact, the direct effects of this chimeric compound have been tested in different normal and tumoral pituitary cell models [8, 18-24]. Recently, our group has demonstrated that treatment with BIM-23A760 reduce hormone secretion and/or expression in a set of somatotropinomas (GHomas), prolactinomas (PRLomas) and corticotropinomas (ACTHomas) [8]. However, these results also showed the existence of two PitNET cell populations that oppositely responded to the treatment of BIM-23A760, an inhibitory- and stimulatory-population [8], suggesting that this compound might not be a successful therapeutic option in some patients with PAs. Moreover, BIM-23A760 has been finally withdrawn from clinical development after discovering a dopaminergic metabolite that accumulates and interferes with the activity of the parent compound *in vivo* [17].

For all these reasons, a new generation of chimeric agonist for SST_2/SST_5/D_2 receptors (named BIM-065), with higher potency, efficacy and safety has been recently designed and developed by IPSEN that may be used for clinical purposes in the future [25, 26]. Therefore, the main aim of this study was to evaluate, for the first time, the direct effects of this new compound on relevant functional parameters (cell viability, apoptosis, hormonal secretion/expression and $[Ca^{2+}]_i$ kinetics) in primary cell cultures from different PitNETs, including ACTHomas, GHomas, non-functioning pituitary adenomas (NFPAs) and PRLomas.

3. Materials and Methods

3.1 Reagents

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. BIM-065 and cabergoline were kindly provided by IPSEN Bioscience (Cambridge, MA, USA) and prepared following the instructions of the company. Octreotide was obtained from GP-Pharm (Barcelona, Spain) and pasireotide was generously provided by Novartis. All compounds were used at 100nM based on previous studies [8, 27, 28].

3.2 Patients, samples and primary cell cultures

Human PitNETs were obtained during trans-sphenoidal surgery from a total of 31 patients: 9 ACTHomas (mean age: 51 [29 – 79] years; 67% women), 9 GHomas (mean age: 48 [29 – 63] years;

44% women), 2 PRLomas (mean age: 25 [20 – 30] years; 50% women) and 11 NFPAs (mean age: 56 [41 – 75] years; 45% women). The appropriate classification of each pituitary sample collected was confirmed by two different methods: examination by expert anatomo-pathologists and by the molecular screening using quantitative real-time PCR (qPCR) as previously described [8, 28-30]. All techniques carried out in this study were conducted in accordance with the ethical standards of the Helsinki Declaration, of the World Medical Association and with the approval of the University of Cordoba/IMIBIC and Ethics Committees from all the Hospitals involved in the study. Informed consent from each patient was obtained.

All human samples were placed in sterile cold medium (S-MEM, Gibco, Madrid, Spain) supplemented with 0.1% BSA, 0.01% L-glutamine, 1% antibiotic-antimycotic solution, and 2,5% 2-(4(2-hydroxyethyl)-1-piperazine)-ethane sulfonic acid (HEPES). The samples were rapidly moved to our laboratory and dispersed into single cells by mechanical and enzymatic disruption and cultured onto different tissue culture plates following the methods and reagents previously described [28, 29].

3.3 Cell line and culturing.

The mouse corticotroph pituitary derived cell line AtT-20/D16v-F2 (ATCC® CRL-1795) was cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) complemented with 10% FBS, 100 U/ml penicillin/streptomycin, 0.024 M of HEPES, and maintained at 37ºC and 5% CO2, under sterile conditions, as previously reported [29]. The passage numbers used for the experiments were ranged between 20 and 25. Additionally, both cell lines were checked for mycoplasma contamination by PCR [31].

3.4 Measurement of cell viability

Cell viability of PitNET cell cultures (10,000 cells/well in 96-well plates) was evaluated every 24h until 72h in response to BIM-065, octreotide, pasireotide or cabergoline using Alamar-blue reagent (Invitrogen, Madrid, Spain). In experiments using AtT-20 cell line, 6,000 cells/well were seeded in 96-well plates. The treatment was daily refreshed after each measure. Reduction of Alamar-blue was quantified using a FlexStation III system (Molecular Devices, Sunnyvale, CA), as previously reported [8, 30, 32].

3.5 Measurement of apoptotic rate

Apoptotic rate was determined by measuring caspase 3/7 activity in response to BIM-065 using the Caspase-Glo 3/7 assay (Promega, Madrid, Spain) according to manufacturer's instructions. Briefly, 25,000 cells/well were plated in a 96-well white microplate and cultured for 24h at 37°C in an atmosphere containing 5% CO_2. Then, cells were incubated for another 24h with BIM-065 and vehicle. After the incubation period, 100µl of Caspase-Glo 3/7 reagent was added to each well and luminescence was measured at room temperature using FlexStation III system (Molecular Devices, Sunnyvale, CA) for 3h.

3.6 Measurement of pituitary hormone release

To evaluate the effect of BIM-065 on pituitary hormone secretion, 150,000 – 200,000 cells/well were used. Media were recollected after 24h of treatment and ACTH, GH, chromogranin-A (CgA) and PRL were evaluated using commercial ELISA kits (reference numbers: EIA-3647, EIA-3552, EIA-4937 and EIA-1291, respectively; DRG, Mountainside, NJ, USA) following the manufacturer's instructions. All the information regarding specificity, detectability, and reproducibility for each of the assays can be accessed at the website of the company.

3.7 Measurement of dynamics of free cytosolic calcium concentration ($[Ca^{2+}]_i$).

To assess the direct effect of BIM-065 on free cytosolic calcium mobilization, 50,000 cells/coverslip were plated and changes in $[Ca^{2+}]_i$ in single cells of all types of PitNETs were measured using fura-2AM (Molecular Probes, Eugene, OR), as previously described [8, 29, 30].

3.8 Measurement of MAPK and PI3K-Akt signaling pathways by western blotting

The mouse corticotroph PitNET derived cell line AtT-20/D16v-F2 (ATCC® CRL-1795) was used to further explore the signaling pathways modulated in response to BIM-065 due to the limited number of primary PitNET cells available for the culture experiments. 500,000 cells were cultured in 6-well plates and incubated for 24h with BIM-065 and vehicle-treated controls, as described previously [30]. Extracted protein samples were incubated with the primary antibodies [p-AKT (Ser47; Ref. CS9271S), Akt (Ref. CS9272), Bcl-2 (Ref. CS3498), p-ERK1/2 (Ref. CS4370), ERK1/2 (Ref. CS154), from Cell Signaling (Danvers, MA, USA)] and the appropriate secondary antibodies (secondary anti-rabbit antibody from Cell Signaling, Danvers, MA, USA), and developed using an enhanced chemiluminescence detection system (GE Healthcare, UK) with dyed molecular weight markers. A densitometric analysis of the bands was carried out with ImageJ software [33]. Relative phosphorylation of ERK1/2 and Akt was obtained from normalization of p-ERK1/2 or p-Akt against the total ERK1/2 or p-Akt, respectively. Bcl-2 was normalized by using total protein loading (ponceau staining).

3.9 RNA isolation, reverse transcription and analysis of gene expression levels by qPCR.

Details of RNA extraction, quantification, reverse-transcription (RT) and qPCR using specific primers included in this study have been previously reported elsewhere by our group [8, 30, 34, 35]. It should be mentioned that since it is not possible to design a specific set of primers for qPCR that only amplified the short isoform of D_2, a set of primers that amplify both, the long and short, isoforms (D_2T) and a set of primers that only amplify the long isoform (D_2L) were used in this study, as previously reported [36]. In addition, new primer sequences were used in the present study to amplify CDKN1B (sense, ATAAGGAAGCGACCTGCAAC and antisense, TTGGGGAACCGTCTGAAA; accession number, NC_000012.12; product size, 88 pb) and CDK2 (sense, GCTCTCACTGGCATTCCTCTT and antisense, GAGGTTTAAGGTCTCGGTGGA; accession number, NC_000012.12; product size, 109 pb). It should be noted that, as previously reported and based on the stringent criteria to maximize specificity and efficiency, the qPCR technique, as applied, can be used to accurately quantify copy numbers for all human transcripts included in this study [36]. A no RT sample was used to ascertain that no detectable genomic DNA was present in the total RNA preparation. Moreover, to control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, the expression level (copy-number) of each transcript was adjusted using a normalization factor (NF) calculated from beta actin (ACTB), hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels.

3.10 Statistical analysis

All data are expressed as mean ± SEM. Statistical differences were assessed by paired parametric t-test or one-way ANOVA followed by Dunnett´s test for multiple comparison (according to normality evaluated by Kolmogorov-Smirnov test). As previously reported [29], to normalize values within each treatment and minimize intragroup variations in the different *in vitro* experiments (i.e. different age of the tissue donor and metabolic environment), the values obtained were compared with vehicle-treated controls (set at 100%). All experiments were performed in a minimum of three independent primary pituitary cultures from different patients (3 – 4 replicates/treatment per experiment), unless otherwise indicated. p-values ≤0.05 were considered statistically significant and a trend for significance was indicated when p-values ranged between >0.05 and <0.1. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software; La Jolla, CA, USA).

4. Results

4.1 Expression profile of SST_2, SST_5 and D_2 and effects of BIM-065 on ACTHomas

SST_2, SST_5 and D_2, the target receptors for BIM-065, were highly expressed in human ACTHomas, with relative order of $D_2T>D_2L>SST_5>>SST_2$ (Fig-1A), suggesting that corticotropinomas represent an appropriate model to test the effect of the new dopastatin compound. We also measured the expression of the two truncated SST_5 variants, SST_5TMD4 and SST_5TMD5, since the presence of these isoforms have been associated to a poor response to SSAs [7, 37]; however, the expression levels of these truncated SST_5 variants were negligible (less than 10 copies) in this cohort of samples.

Incubation of cultured ACTHoma cells with different doses of BIM-065 (10^{-9} to 10^{-6} M) revealed clear inhibitory effects on cell viability (Fig-1B). Interestingly, the four doses employed reduced cell viability at similar levels after 48-72h of incubation. It should be noted that, due to the limited number of cells available for the culture experiments, we could not test a wider range of doses; however, we had the opportunity to expand the doses employed in one ACTHoma cell preparation, in which a clear dose-response reduction on cell viability was observed at lower concentrations of BIM-065 (10^{-13} to 10^{-9} M; Supplementary Figure 1A). Specifically, BIM-065 does not seem to significantly alter cell viability at 10^{-13} or 10^{-11} M, whereas, as previously observed (Fig-1B), it clearly reduced this parameter at 10^{-9} M after 72h of incubation (Supplementary Figure 1A). Based on these results and, in order to compare the effects of BIM-065 with previously selected, efficacious doses of SSAs (i.e. octreotide and pasireotide) and BIM-23A760 (the first generation dopastatin compound) in PA cell cultures [8, 28], we selected the 10^{-7} M dose in further experiments.

Our results indicated that BIM-065 and octreotide produced a comparable significant reduction on cell viability in ACTHoma cell cultures (31.1% vs. 26.1%); however, pasireotide did not significantly alter cell viability (Fig-1C). Interestingly, we could also compare the effects of BIM-065 and octreotide at a lower dose (10^{-9} M) in an ACTHoma cell preparation, and the results clearly indicated that BIM-065 seems to be more effective than octreotide in reducing cell-viability after 72h of incubation (41% vs. 10%, respectively; Supplementary Figure 1B). In addition, we observed that incubation with BIM-065 (10^{-7} M) increased apoptosis after 24h compared with vehicle-treated controls (Fig-1D). Moreover, BIM-065 also appeared to decrease ACTH release in all ACTHomas analyzed, although this difference did not reach statistical significance probably due to the limited number of samples (p=0.07; Fig-1E); whereas, it did not alter mRNA expression levels of POMC (ACTH-precursor), somatostatin and dopamine receptors (SST_2, SST_5, D_2T, D_2L; Fig-1F) or tumor markers commonly associated with progression and aggressiveness of PitNETs such as PTTG1, CDKN1B and CDK2 [38-40] (Supplementary Figure 5A).

We also analyzed the direct effects of BIM-065 on $[Ca^{2+}]_i$ dynamics in single cells, since this is a key second messenger involved in the regulation of pituitary cell physiology and is closely related to hormone secretion through the modulation of secretory vesicle release [30, 41, 42]. This showed that BIM-065 treatment evoked a rapid inhibitory effect in the kinetics of $[Ca^{2+}]_i$ in all ACTHomas cases analyzed (3/3), exhibiting an averaged 26.4% of reduction in 44% of responsive corticotropinoma cells (Fig-1G). Finally, to further test the capacity of BIM-065 to induce functional responses in pituitary tumor cells, we measured the levels of phosphorylation of several components of two important signaling pathways (MAPK and PI3K-Akt) in the PitNET derived cell line AtT-20 (Fig-2). As previously observed in primary ACTHoma cell cultures, treatment with BIM-065 (10^{-7} M) markedly reduced cell proliferation in cultured AtT-20 cells as compared to vehicle-treated controls (Fig-2A). Interestingly, short-term incubation with BIM-065 (10 min) clearly increased p-Akt (p=0.004), which was followed by a clear reduction of the anti-apoptotic protein Bcl-2 (Fig-2B). In contrast, long-term incubation with BIM-065 (24h) up-regulated p-ERK1/2 levels, although this latter effect did not reach statistical significance (p=0.1; Fig-2B).

4.2 Expression profile of SST_2, SST_5 and D_2 and effects of BIM-065 on GHomas

Similar to ACTHomas, SST_2, SST_5 and D_2 were highly expressed in human GHomas, with relative order of $D_2T>D_2L>SST_5>>SST_2$ (Fig-3A). This expression profile is consistent with previous results evaluating

and comparing the mRNA levels and protein content [43, 44], therefore supporting that somatotropinomas also represent an appropriate model to test the effect of BIM-065. It should be mentioned that the expression levels of the truncated SST_5 variants were also negligible in this set of samples.

Specifically, treatment with BIM-065 (10^{-7} M) significantly decreased cell viability in GHoma cell cultures after 72h of incubation. Our data also revealed that the efficacy of BIM-065, octreotide and pasireotide to reduce cell viability was comparable in GHoma cell cultures (35.6%, 24.7%, 41.8%, respectively; Fig-3B) with respect to vehicle-treated control. BIM-065 also increased apoptosis (Fig-3C), and strongly reduced GH secretion after 24h of incubation (Fig-3D). In line with this, we also had the opportunity to compare the effects of BIM-065 vs. octreotide/pasireotide (10^{-7}M) in two independent GHoma cell cultures and found that all the compounds inhibited GH release (Supplementary Figure 2).

Treatment with BIM-065 did not alter the mRNA expression levels of GH or other genes of interest mentioned above [SST_2, SST_5, D_2T, D_2L (Fig-3E); PTTG1, CDKN1B and (Supplementary Figure 5B)], except for CDK2, whose expression increased in response to BIM-065 compared to vehicle treated-control (Supplementary Figure 5B). Finally, BIM-065 treatment clearly reduced $[Ca^{2+}]_i$ levels (32.74% of reduction) in 2 out of the 3 GHomas analyzed, affecting the 30% of cells (Fig-3F).

4.3 Expression profile of SST_2, SST_5 and D_2 and effects of BIM-065 on NFPAs

NFPAs expressed high levels of SST_2, SST_5 and D_2, and negligible levels of truncated SST_5TMD4 and SST_5TMD5, with relative order of $D_2T>D_2L>>SST_2>SST_5$ (Fig-4A). Remarkably, in contrast to that found with ACTHomas and GHomas, none of the NFPAs analyzed in this study responded to BIM-065 in terms of cell viability (Fig-4B; Supplementary Figure 3). Conversely, similar to ACTHomas and GHomas, NFPA cells were clearly responsive to BIM-065 in terms of increase in apoptosis (Fig-4C) and reduce of CgA-secretion (Fig-4D) after 24h of incubation. Moreover, as we observed with ACTHomas and GHomas, treatment with BIM-065 did not produce any significant alteration in the mRNA expression levels of gonadotropin pituitary hormones (FSHB, LHB or CGA), SST_2, SST_5, D_2T, D_2L (Fig-4E) or of the tumor markers PTTG1 or CDK2, while CDKN1B expression slightly decreased in response to BIM-065 compared to vehicle treated-control (Supplementary Figure 5C). Finally, BIM-065 treatment also reduced $[Ca^{2+}]_i$ levels (30.18% of reduction) in 2/3 NFPAs analyzed but only in a limited percentage of NFPA cells (27%) as compared to ACTHoma and GHoma cells (43.50% or 30.43%, respectively; Fig-4F).

4.4 Expression profile of SST_2, SST_5 and D_2 and effects of BIM-065 on PRLomas

We also had the opportunity to analyze the expression levels of SST_2, SST_5, D_2s and truncated SST_5 variants in two available PRLoma samples and to test the response to BIM-065. Specifically, PRLoma cells expressed high levels of D_2 compared with SST_2 and SST_5 (and negligible levels of truncated SST_5 variants), with relative order of $D_2T>D_2L>>>>SST_5$ in PRLoma-1 and $D_2T>D_2L>>>>SST_2>$ in PRLoma-2 (Supplementary Figure 4A). BIM-065 decreased cell viability at all the doses tested after 72h of incubation (Supplementary Figure 4B) and this reduction was seemingly more pronounced than the effect of cabergoline (34.92% vs 15.4%, respectively; Supplementary Figure 4C). A decrease of PRL secretion was also observed in response to BIM-065 compared with vehicle-treated control after 24h of incubation (Supplementary Figure 4D). Similar to than previously observed in other cultured PitNET cell types, the expression levels of PRL, SST_2, SST_5, D_2T and D_2L were not significantly altered in response to BIM-065 (Supplementary Figure 4F). Moreover, BIM-065 treatment did not alter $[Ca^{2+}]_i$ kinetics in the PRLoma analyzed (Supplementary Figure 4E).

5. Discussion/Conclusion

In the present study, we have tested, for the first time and in a representative set of distinct PitNETs subtypes, the actions of a new peptidic chimeric compound, called BIM-065. BIM-065 has been designed with the same affinity to bind SST_2, higher affinity to bind SST_5 and slightly smaller affinity to bind D_2 compared to the previous chimeric compound known as BIM-23A760 (binding affinity of BIM-065 in nM: 0.03 for SST_2; 0.5 for SST_5 and 27.2 for D_2 [17, 25, 26]). Moreover, this second-generation dopastatin molecule has been designed with an improved chemical structure to avoid the generation of metabolites with potent dopaminergic activity as the one found to accumulate in patients enrolled in Phase IIb study in response to BIM-23A760 [17], and also, to avoid the paradoxical stimulatory responses found in a small but relevant proportion of pituitary tumor cell populations after treatment with this latter compound [8].

Of particular novelty are the results showing that BIM-065 decreases cell viability, increases apoptosis and inhibits ACTH secretion in corticotropinomas, without significantly altering mRNA expression levels of the genes of interest at the times evaluated. To our knowledge, this is the first time that the effect of a chimeric compound is evaluated in detail in ACTH-secreting PitNETs. Although BIM-065 induced a comparable reduction of cell viability than octreotide at 10^{-7} M, the results achieved at 10^{-9} M revealed a higher reduction of cell viability in response to BIM-065. Nevertheless, this observation should be taken with caution, as this comparison could only be tested in one ACTHoma. In contrast, pasireotide did not exert an inhibitory effect on cell proliferation despite the high expression levels of SST_5 and also SST_2, which might suggest that additional mechanisms distinct from the mere presence of a given receptor are required to exert an anti-proliferative effect. Indeed, several reports have described that the expression levels of other proteins such as filamin A, β-arrestins or E-cadherin might be important for SSAs to exert their antitumor actions in pituitary tumors [45-49]. Interestingly, results of a previous study from our group demonstrated a decrease on ACTH secretion in response to BIM-23A760 in one ACTHoma analyzed [8], which is in accordance with the results of this report. In the same line, we observed a reduction on $[Ca^{2+}]_i$ levels in all ACTHomas tested in response to BIM-065, which is also in accordance with the $[Ca^{2+}]_i$ response observed in the previous study. Indeed, the reduction of $[Ca^{2+}]_i$ levels was similar in both studies (26.4% of decrease in response to BIM-065 vs 27% of decrease observed in response to BIM-23A760) but the percentage of responsive cells was higher with BIM-065 than with BIM-23A760 (43.50% vs 24.0%, respectively) [8]. Of note, in contrast with the previous study, we did not observe any stimulatory effect in response to BIM-065 in the ACTHomas tested. In addition, we also demonstrate that treatment with BIM-065 increased p-Akt and p-ERK1/2 levels and decreased Bcl-2 in AtT-20 cells. These two pathways are tightly linked to cell growth, proliferation and survival in tumor pathologies, including PitNETs [50, 51], albeit they displayed a distinct temporal course: p-Akt increased acutely, at 10 min, while p-ERK1/2 levels raised after long-term, 24h-incubation. A similar stimulatory response in ERK1/2 levels has been previously reported in PitNET cells after treatment with BIM-23A760 and the dopaminergic agonist BIM-53097, which was mainly associated to an activation of the dopaminergic signaling through D_2 that contributed to the antiproliferative effects of the two compounds in NFPA cell cultures [22]. Therefore, our results might suggest that the effects of BIM-065 on p-ERK1/2 and p-Akt proteins might be associated to a preferential activation of the D_2/dopaminergic-signaling. Moreover, the decrease in Bcl-2 levels support the increase of apoptosis observed in response to BIM-065 in PitNETs. Therefore, the data presented provide novel evidence regarding how this new compound regulates some key intracellular signaling pathways in PitNETs cells. It should be noted that although a side-by-side study comparing BIM-065 and BIM-23A760 might have been interesting to make direct comparisons between both dopastatin compounds, the limitation of PitNET samples and the fact that BIM-23A760 was withdrawn from clinical development, prompted us to disregard this option and to implement the direct, side-by-side comparison of BIM-065 with treatments currently used in medical practice (octreotide and pasireotide).

Results on GH-secreting PitNETs revealed that BIM-065 also induced profound direct actions on this tumor type, where we observed a higher reduction of cell viability and a comparable increase of apoptosis compared with our previous results with BIM-23A760 in somatotropinomas [8]. In the case of GH secretion, we observed a 53.2% of reduction of GH release, which is more striking than the decrease produced in response to BIM-23A760 or other chimeric compounds (SST_2-SST_5 or SST_2-D_2) in somatotropinomas or GH3 pituitary cell line [8, 18, 24, 52]. These results are in agreement with a very recent report showing that BIM-065 can reduce GH and IGF1 levels in healthy male volunteers with a safety and tolerability profile only limited by orthostatic hypotension [53]. On the other hand, treatment with BIM-065 was not accompanied by decreased GH mRNA levels after long-term exposition (24h) in human GH-secreting PitNETs, which is in contrast to our previous results with BIM-23A760 [8], but compares well with the results reported by Gruszka et al. in response to BIM-23A760 and BIM-23A761 [24]. Comparison of GH reduction between BIM-065 and SSAs did not show any relevant difference in the cases analyzed. Nevertheless, further experiments testing different BIM-065 doses and in additional tumor preparations will be necessary to unequivocally establish whether BIM-065 is more potent and efficacious than SSAs in somatotropinomas. An initial analysis of the possible mechanisms involved in the functional response observed in these tumors revealed that the new dopastatin evoked a clear reduction on $[Ca^{2+}]_i$ levels, which also agrees with the predominant inhibitory responses observed by our group in the face of BIM-23A760 [8]; yet, in clear discrepancy with that observed previously for BIM-23A760 in somatotropinomas [8], but in line with our present results in ACTHomas, we did not detect herein any stimulatory response from somatotropinoma cells.

It is currently rare to have access to PRLoma samples for *in vitro* testing, given, fortunately, to the frequent success of dopamine agonists treatment regimens to control or even cure this type of tumors. However, in the course of this study we had the opportunity to test this compound on two PRL-secreting PitNETs. Interestingly, we observed clear differences in the response to BIM-065 and cabergoline in terms of cell viability between the two PRLomas analyzed, which could be due to their differential receptor expression pattern. Thus, the tumor that was highly responsive to BIM-065 expressed higher levels of SST_5 compared to SST_2 and higher levels (and similar between them) of both isoforms of D_2, which is a comparable expression pattern to that found in the rest of functioning PitNETs analyzed. Furthermore, these data are in line with previous results reporting that dopaminergic contribution is more important for the response to chimeric compounds, such as BIM-23A760, than the expression of SST_2 [54]. Consistent with previous results observed with chimeric compounds [8, 24], a profound decrease on PRL release was detected in response to BIM-065; whereas, in contrast with the data reported so far in human PRLomas, rat pituitary cell cultures and MMQ cell line [8, 24, 52], no changes were found on PRL mRNA levels. Nonetheless, our results showing that BIM-065 is able to decrease PRL release compare favorably with a very recent report showing that this compound inhibited PRL levels in healthy male volunteers [53]. Obviously, additional experiments are necessary to confirm/complement the results obtained herein, and to explore the signaling pathways involved, particularly given the unexpected observation that the effects observed in PRLomas did not seem to be accompanied by parallel changes in $[Ca^{2+}]_i$ dynamics.

In contrast to previous results from our group and others with other dopastatins [8, 21], NFPA primary cell cultures did not show relevant alterations in terms of cell viability in response to BIM-065. Yet, surprisingly, the treatment with BIM-065 did induce a clear increase on apoptotic rate and a significant decrease in CgA in all cases tested. Although the vast majority of observations suggest that the cell proliferation and programmed cell death are effectively coupled [55, 56], there are several studies showing an imbalance between cell proliferation/survival index and apoptosis in pathological conditions such as breast, lung or colorectal cancer [57-59]. Nevertheless, this observation is both, clinically relevant for this difficult-to-treat type of tumor, and biologically intriguing, and therefore further studies are warranted to explore the reasons why cell proliferation and apoptosis are apparently uncoupled in response to BIM-065 in NFPA primary cell cultures.

In summary, our data provide compelling evidence demonstrating that BIM-065 can directly and profoundly alter cell function and behavior in the most relevant subtypes of PitNETs. While not universal for all tumor types, BIM-065 acted reducing cell viability, likely by increasing apoptosis, and inhibiting hormone secretion, mostly by reducing $[Ca^{2+}]_i$ levels. Importantly, in contrast to previous dopastatin, treatment with BIM-065 did not evoke any stimulatory action in the tumor cells analyzed, while the proportion of responsive tumors/cells was higher with this compound than with BIM-23A760, suggesting that this novel dopastatin is a more efficacious and specific chimeric compound that could become a useful tool in the future treatment of PitNETs. Indeed, this compound could be used to treat patients harboring NFPAs without a clear indication of surgical resection, patients with a tumor residue or with hormone hypersecretion persistence after transsphenoidal surgery or to control hormone hypersecretion and reduce tumor growth before surgery. In any case, further studies should be implemented to confirm and expand the original results provided herein, and a randomized clinical trial comparing BIM-065 with the pharmacological options currently available should be performed. Thus, this new generation chimeric compound may hopefully help to enhance the currently scarce pharmacological arsenal for the treatment of patients harboring PitNETs.

8. Statements

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8.2. Statement of Ethics

All techniques carried out in this study were conducted in accordance with the ethical standards of the Helsinki Declaration, of the World Medical Association and with the approval of the University of Cordoba/IMIBIC and Ethics Committees from all the Hospitals involved in the study. Informed consent from each patient was obtained.

8.3. Disclosure Statement

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8.5. Author Contributions

Conception and design of research: M.C.V.-B., T. L., M.D.C., J.P.C., R.M.L.; Performed experiments: M.C.V.-B., F.L.-L., A.C.F.-F, A. D. H.-M; Analyzed data: M.C.V.-B., A.C.F.-F., J.P.C., R.M.L.; Interpreted results: M.C.V.-B., A.C.F.-F., M.D.G., M.D.C., J.P.C., R.M.L.; Prepared figures: M.C.V.-B., A.C.F.-F., R.M.L.; Acquisition of clinical/pathological data and samples: M.A.-G., E.V.-M., A.D.H.-M., C.B.-A., J.S., A.S.; Wrote the manuscript: M.C.V.-B., M.D.G., J.P.C., R.M.L.; Critically revised the manuscript and approved final version: M.C.V.-B., F.L.-L., M.A.-G., A.C.F.-F, E.V.-M., A. D. H.-M., C.B.-A., J.S., T.L., M.D.G., A.S., M.D.C., J.P.C., R.M.L.

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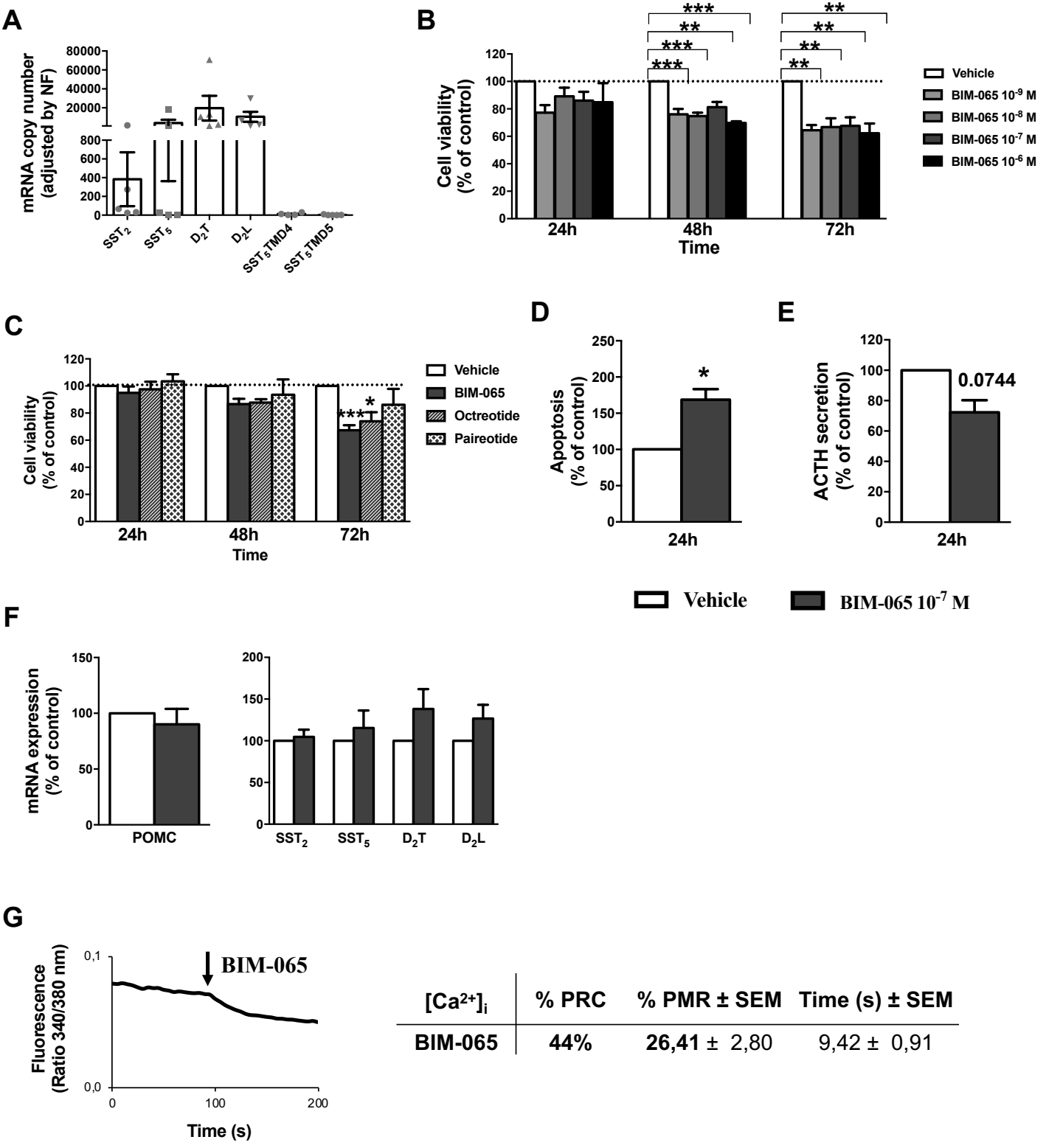
10. Figure Legends

Figure 1: ACTH-secreting adenomas. (A) Expression profile of SST_2, SST_5, D_2 (total and long isoforms), SST_5TMD4 and SST_5TMD5 (n=5). (B) Dose-response experiment of cell viability in response to BIM-065 (10^{-9} to 10^{-6} M; n=3). (C) Effect of BIM-065, octreotide and pasireotide on cell viability (10^{-7} M; n=3-6; 24 – 72h treatment). (D) Effect of BIM-065 (n=3) on apoptosis (24h treatment). (E) Measurement of ACTH secretion (24h treatment; n=3). (F) mRNA expression levels of key genes in response to BIM-065 measured by qPCR and adjusted by normalization factor (NF) (n=4). (G) Summarized table and representative profile of $[Ca^{2+}]_i$ kinetics assay in response to BIM-065 (n=3). PRC, proportion of responsive cells showing changes in $[Ca^{2+}]_i$ in response to the treatment; PMR, percentage of maximum response; and time, time of response to treatment administration. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* p<0.05; ** p<0.01; *** p<0.001) indicate statistically significant differences.

Figure 2: AtT-20 corticotrope cell line. (A) Effect of BIM-065 (10^{-7} M; n=5) of cell proliferation (24 – 72h treatment), measured by Alamar-blue reduction. (B) Representative Western Blots and quantification of levels of p-Akt/total Akt, Bcl-2/ponceau and p-ERK1/2/ total ERK1/2 in response to BIM-065 (10^{-7} M; n=3). Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* p<0.05; ** p<0.01; *** p<0.001) indicate statistically significant differences.

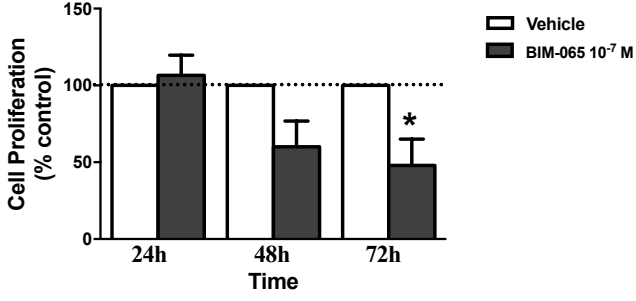
Figure 3: GH-secreting adenomas. (A) Expression profile of SST_2, SST_5, D_2 (total and long isoforms), SST_5TMD4 and SST_5TMD5 (n=4). (B) Effect of BIM-065, octreotide and pasireotide on cell viability (10^{-7} M; n=4; 24 – 72h treatment). (C) Effect of BIM-065 (n=3) on apoptosis (24h treatment). (D) Measurement of GH secretion (24h treatment; n=4). (E) mRNA expression levels of key genes in response to BIM-065 were measured by qPCR and adjusted by normalization factor (NF) (n=3). (F) Summarized table and representative profile of $[Ca^{2+}]_i$ kinetics assay in response to BIM-065 (n=3). PRC, proportion of responsive cells showing changes in $[Ca^{2+}]_i$ in response to the treatment; PMR, percentage of maximum response; and time, time of response to treatment administration. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* p<0.05; ** p<0.01; *** p<0.001) indicate statistically significant differences.

Figure 4: Non-functioning pituitary adenomas. (A) Expression profile of SST_2, SST_5, D_2 (total and long isoforms), SST_5TMD4 and SST_5TMD5 (n=3). (B) Effect of BIM-065 on cell viability (10^{-7} M; n=8; 24 – 72h treatment), measured by Alamar-blue reduction. (C) Effect of BIM-065 (n=3) on apoptosis (24h treatment), measured by Caspase-Glo 3/7 assay. (D) Measurement of Chromogranin A secretion (24h treatment) (n=3), determined by commercial ELISA kit. (E) mRNA expression levels of key genes in response to BIM-065 were measured by qPCR and adjusted by normalization factor (NF) (n=3). (E) Summarized table and representative profile of $[Ca^{2+}]_i$ kinetics assay in response to BIM-065 (n=3). PRC, proportion of responsive cells showing changes in $[Ca^{2+}]_i$ in response to the treatment; PMR, percentage of maximum response; and time, time of response to treatment administration. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* p<0.05; ** p<0.01; *** p<0.001) indicate statistically significant differences.



$[Ca^{2+}]_i$	% PRC	% PMR ± SEM	Time (s) ± SEM
BIM-065	44%	**26,41** ± 2,80	9,42 ± 0,91

A


Cell Proliferation (% control)
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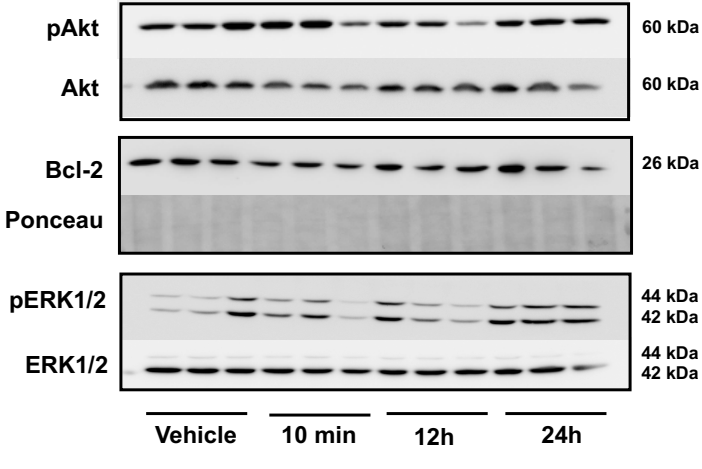
BIM-065 10-7 M

B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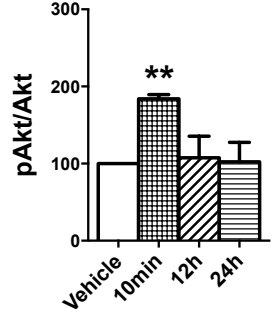
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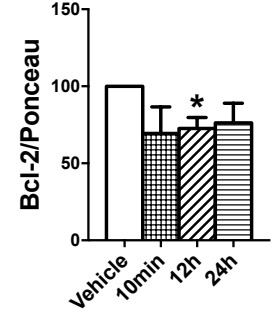
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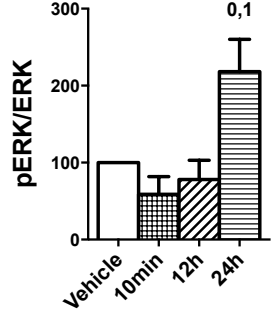
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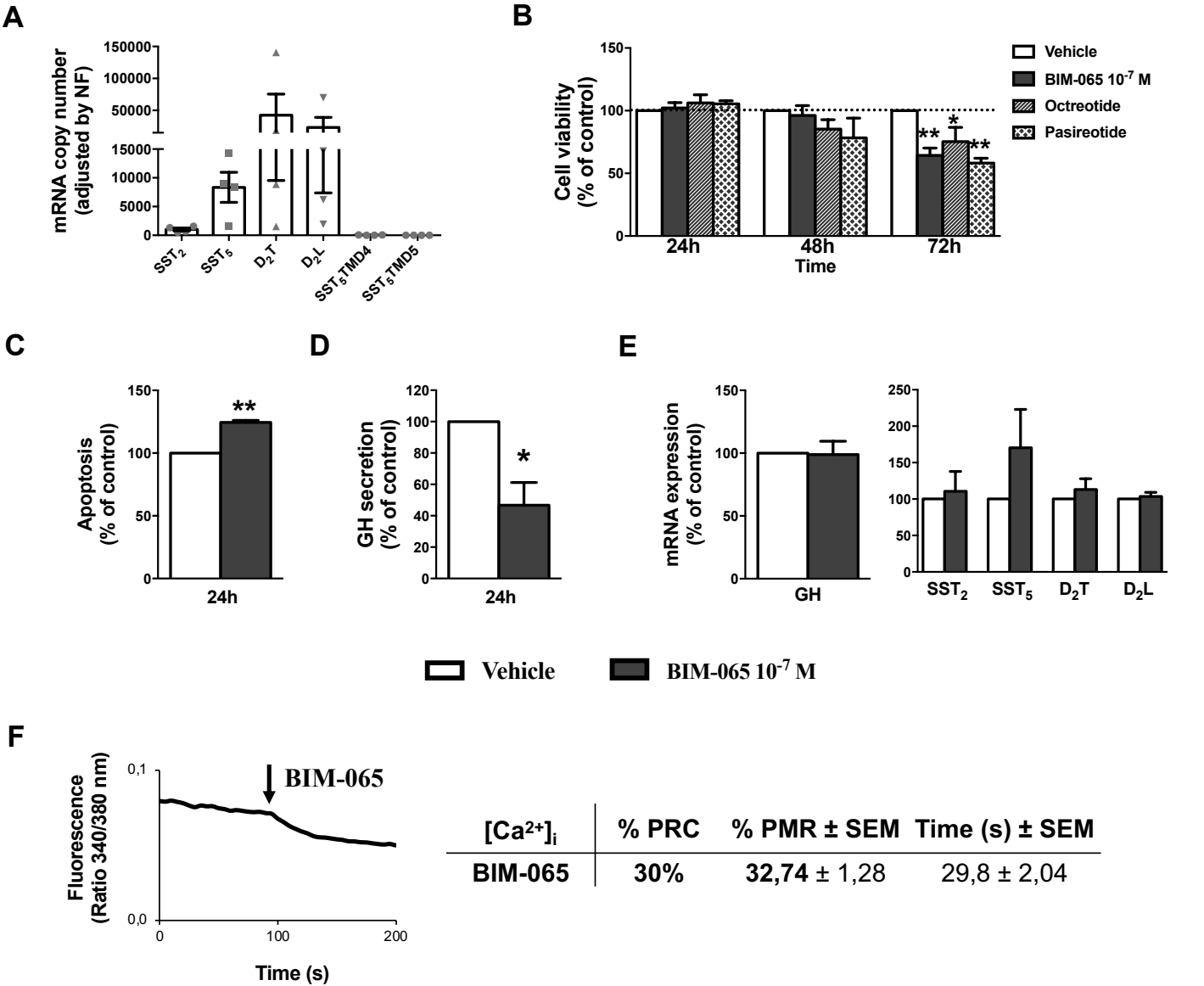
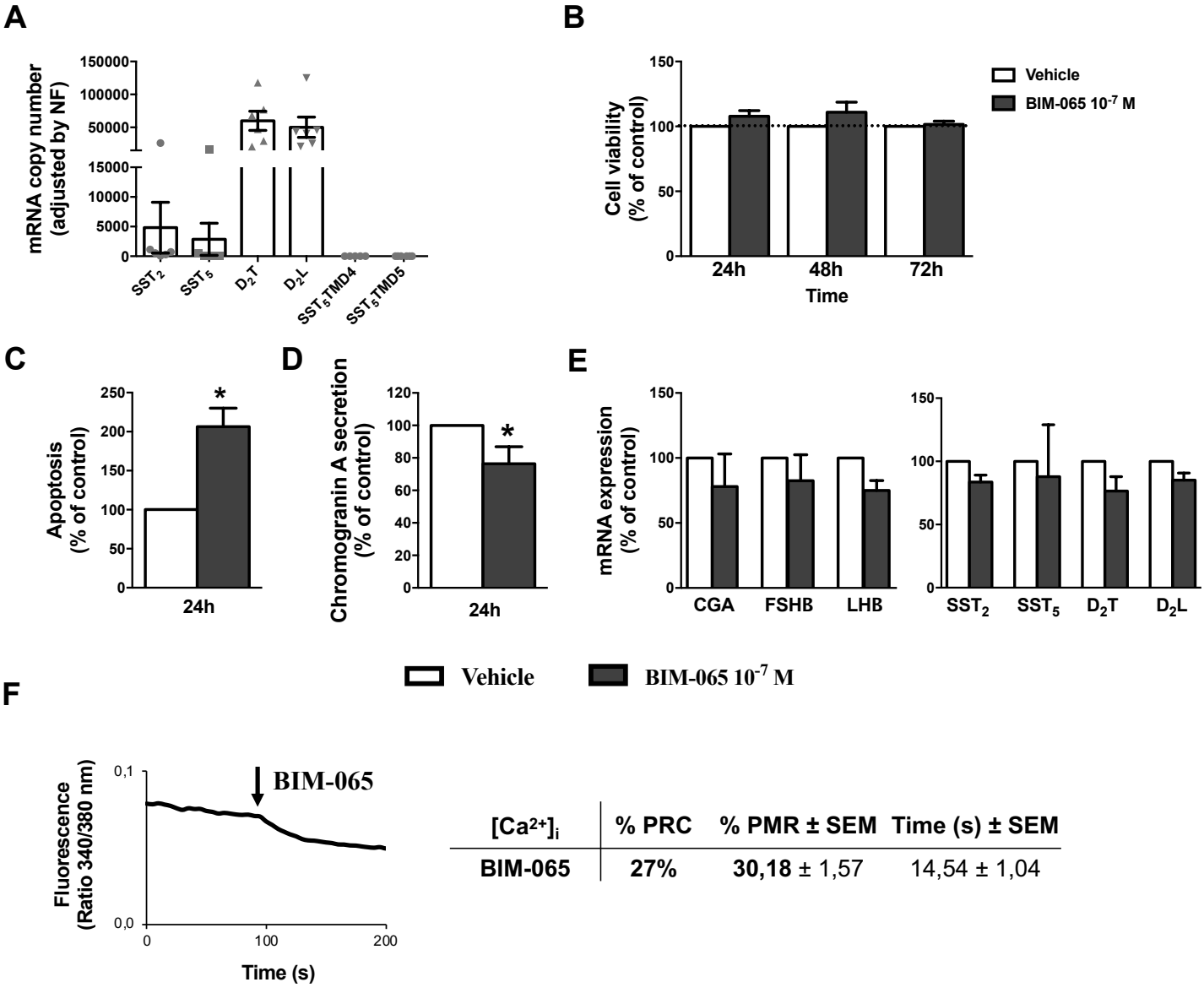
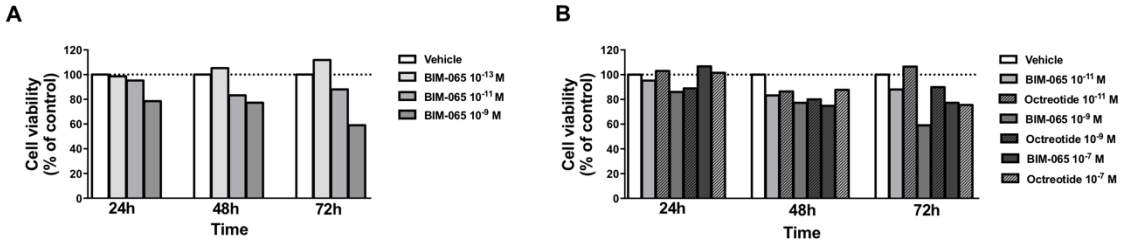


$[Ca^{2+}]_i$	% PRC	% PMR ± SEM	Time (s) ± SEM
BIM-065	30%	**32,74** ± 1,28	29,8 ± 2,04

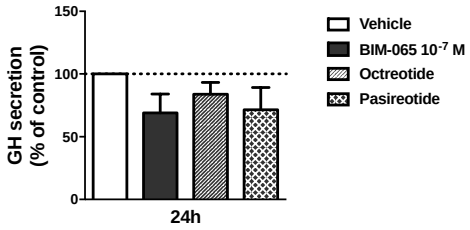


$[Ca^{2+}]_i$	% PRC	% PMR ± SEM	Time (s) ± SEM
BIM-065	27%	**30,18** ± 1,57	14,54 ± 1,04

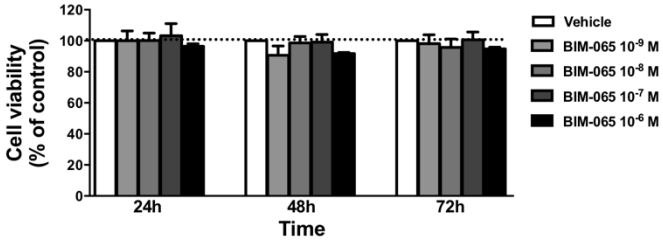
Supplementary Figure 1: ACTH-secreting adenomas. (A) Dose-response experiment of cell viability in response to BIM-065 (10^{-13} to 10^{-9} M; n=1), measured by Alamar-blue reduction. (B) Comparison of dose-response experiment between BIM-065 and octreotide on cell viability (10^{-11} to 10^{-7} M; n=1), measured by Alamar-blue reduction. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. In cases where less than three experiment were carried out, no significance tests were performed.



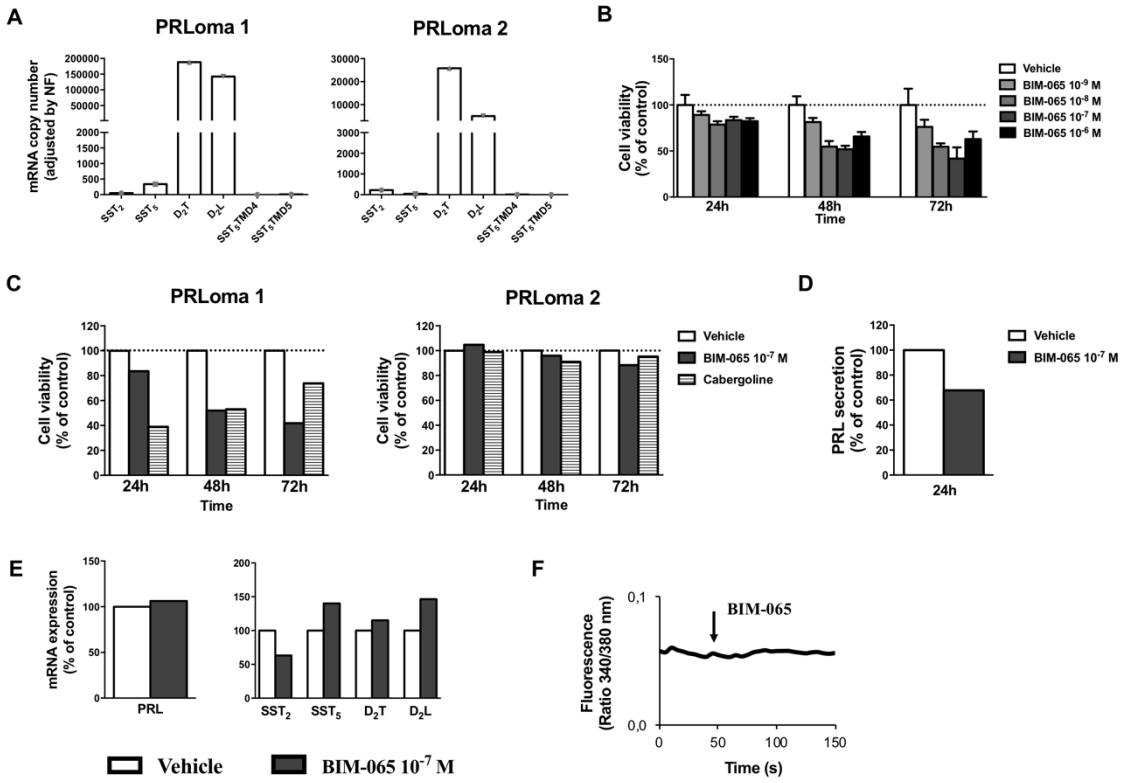
Supplementary Figure 2: GH-secreting adenomas. Direct effect of BIM-065, octreotide and pasireotide on GH secretion (24h treatment; n=2), determined by commercial ELISA kit. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. In cases where less than three experiment were carried out, no significance tests were performed.



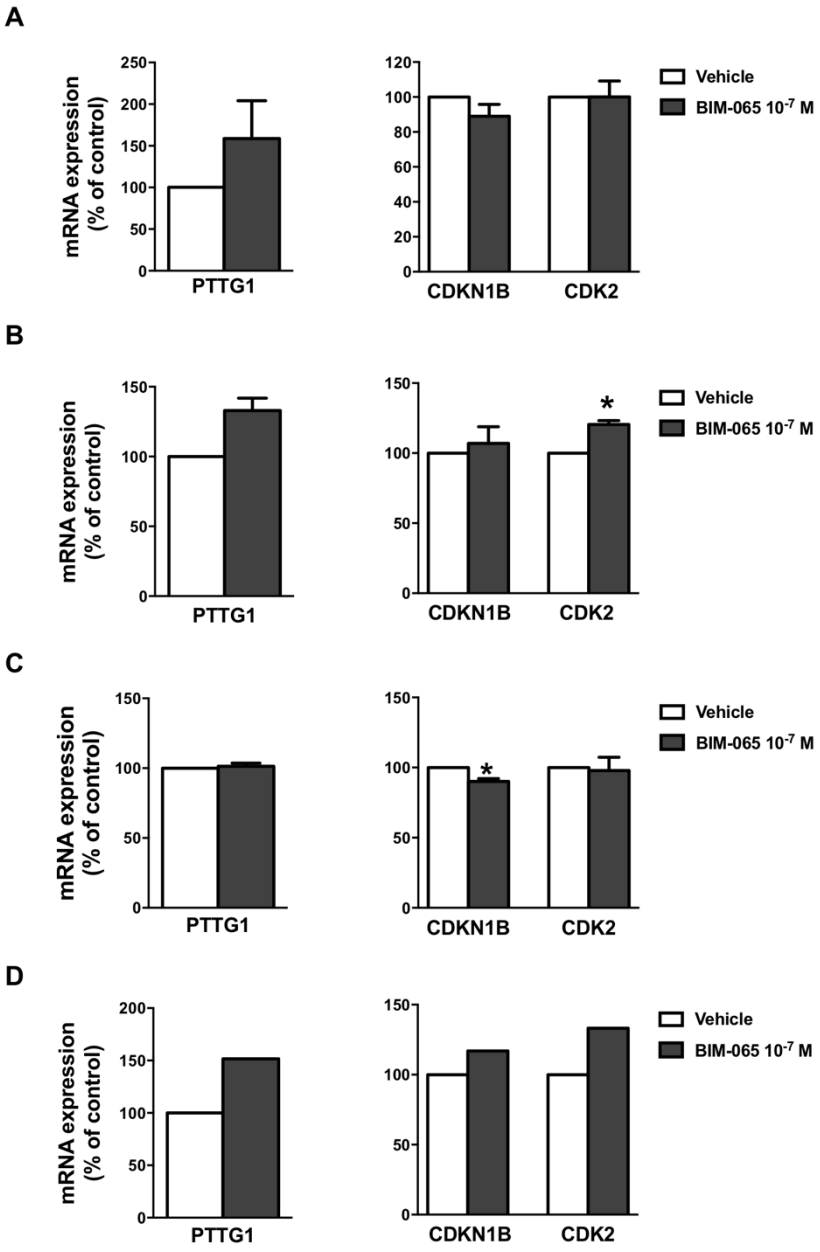
Supplementary Figure 3: Non-functioning pituitary adenomas. Dose-response experiment of cell viability in response to BIM-065 (10^{-9} to 10^{-6} M; n=3), measured by Alamar-blue reduction. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM.



Supplementary Figure 4: PRL-secreting adenomas. (A) Expression profile of SST_2, SST_5, D_2 (total and long isoforms), SST_5TMD4 and SST_5TMD5 of two different PRLomas. (B) Dose-response experiment of cell viability in response to BIM-065 (10^{-9} to 10^{-6} M; n=1), measured by Alamar-blue reduction. (C) Effect of BIM-065 and cabergoline on cell viability (10^{-7} M; 24 – 72h treatment) in two different PRLomas, measured by Alamar-blue reduction. (D) Measurement of PRL secretion (24h treatment) (n=1), determined by commercial ELISA kit. (E) mRNA expression levels of key genes in response to BIM-065 were measured by qPCR and adjusted by normalization factor (NF) (n=1). (F) Representative profile of $[Ca^{2+}]_i$ kinetics assay in response to BIM-065 (n=1). Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. In cases where less than three experiment were carried out, no significance tests were performed.



Supplementary Figure 5: Expression profile of tumor markers (PTTG1, CDKN1B and CDK2) in response to BIM-065 in different pituitary adenomas, measured by qPCR and adjusted by normalization factor (NF). (A) ACTH-secreting PitNETs (n=4); (B) GH-secreting PitNETs (n=3); (C) Non-functioning pituitary adenomas (n=3) (D) PRL-secreting PitNETs (n=1). Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* $p<0.05$) indicate statistically significant differences. In cases where less than three experiment were carried out, no significance tests were performed.



Splicing machinery is dysregulated in pituitary neuroendocrine tumors (PitNETs) and associated with aggressiveness features

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Short title: Dysregulation of splicing machinery in PitNETs.

Keywords: splicing, spliceosome, pituitary neuroendocrine tumors, pladienolide.

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ABSTRACT

Pituitary neuroendocrine tumors (PitNETs) comprise approximately 15% of all brain tumors and the majority of them have a sporadic origin. Although some studies have explored the mutational landscape of PitNETs, there is evidence that additional mechanisms would have to contribute relevantly to the development and behavior of these tumors. Recent studies suggest that altered alternative splicing and, consequently, appearance of an abnormal pattern of splicing and even of aberrant splicing variants, represent a common molecular feature of most tumor pathologies. Moreover, the spliceosome machinery is emerging as an attractive therapeutic target to treat tumor pathologies, and, for instance, inhibition of SF3b1 using pladienolide-B has been shown to exert promising antitumor properties. Therefore, we aimed to analyze the expression levels of selected splicing machinery components in 261 PitNETs [138 GHomas/90 non-functioning PitNETS (NFPTs)/24 ACTHomas/9 PRLomas]. Additionally, we evaluated the direct effects of pladienolide-B in cell-proliferation/viability and hormone secretion in human PitNETs cell cultures and pituitary model cell-lines (AtT-20 and GH3). Results revealed a severe dysregulation of the expression levels of splicing machinery components in all the PitNET-subtypes compared to normal pituitaries, which provided unique fingerprints of splicing components that accurately discriminate between normal and tumor tissue in each of the PitNET-subtypes analyzed. Results also identified several components commonly dysregulated in all PitNET-subtypes. Functional assays demonstrated that pladienolide-B markedly reduced cell-proliferation/viability and GH secretion in PitNET cell-cultures and cell-lines. Altogether, the present results provide novel, compelling evidence to propose that the splicing machinery is severely and distinctly dysregulated in the main PitNET-subtypes. This discovery opens a new window to investigate the plausible contribution of splicing dysregulation and its subsequent outcomes to pituitary tumorigenesis, and to assess the potential value of specific splicing machinery components as novel diagnostic/prognostic tools in these pathologies. Furthermore, our study unveils splicing, particularly SF3b1, as a novel actionable therapeutic point that can be targeted by Pladenolide-B to combat PitNETs.

INTRODUCTION

Pituitary neuroendocrine tumors (PitNETs), formerly referred to as pituitary adenomas, are more abundant than often thought, for they represent approximately 15% of all brain tumors and have an estimated prevalence that ranges from 1 in 865-2,688 people [1, 2]. Likewise, PitNETs have been classically considered as a benign pathology because they rarely metastatize, thus the term adenomas. However, the great variety of clinical behaviors accompanying these pathologies, coupled to their diverse and severe associated comorbidities and increased mortality led the "International Pituitary Pathology Club" to propose, in a recent consensus, a reclassification of pituitary tumors and to establish the nomenclature of "pituitary neuroendocrine tumors (PitNETs)" instead of "pituitary adenomas" [3].

Interestingly, the vast majority of PitNETs have a sporadic origin, whereas only a small percentage (5%) is due to familial tumor syndromes [4, 5]. Moreover, recent studies exploring the genomic landscape of PitNETs confirmed and extended earlier studies by reporting that all major tumor subtypes studied present a relatively low number of somatic mutations per tumor, and that there are scarce recurrent mutations, none of which is commonly found across PitNET subtypes [6, 7]. Overall, these genomic analyses, albeit highly informative and valuable, support the contention that mutations and purely genetic alterations alone would not fully explain PitNET tumorigenesis, and therefore, that alternative oncogenic events, including epigenetic alterations [8] or miRNAs [9, 10], should be explored further to understand their actual contribution in this regard. Indeed, the primary initiating cause of PitNETs development and possible the existence of general and distinctive signatures and molecular elements in this heterogeneous pathology is still under debate [4, 5, 11-14].

In this scenario, an emerging body of evidence indicates that altered alternative splicing and its consequent outcome, i.e. the appearance of abnormal patterns of splicing and even that of aberrant splicing variants, represents a common feature across most tumor pathologies, including PitNETs [15-22]. Alternative RNA splicing is a common post-transcriptional (i.e. epigenetic) mechanism that provides a valuable source of biological versatility under physiological circumstances for most eukaryotic genes (>95%) [23]. The intracellular

machinery that catalyzes and thereby controls the process of alternative splicing is the spliceosome, a ribonucleoproteic complex that recognizes specific sequences that determine the precise localization of the exon-intron junctions [24]. This complex machinery, organized into two systems, the major and the minor spliceosome, is comprised by structural proteins, splicing factors (SFs), RNA-dependent ATPase/helicases, and other regulatory proteins [25, 26]. All these elements cooperate in a highly dynamic fashion to finely regulate the splicing process [27].

Alterations of spliceosome function can compromise the normal splicing process of an ample range of genes, thus originating the appearance of multiple, often aberrant splicing variants, which could be directly associated with the development/progression of tumor pathologies [17, 18, 21, 22, 28]. Indeed, results from our group have demonstrated that oncogenic splicing variants from somatostatin and ghrelin systems (SST_5TMD4/5 and In1-ghrelin) are poorly expressed in normal tissue but highly expressed in neuroendocrine tumors (NETs), including PitNETs [17-20, 29, 30], where they increase aggressiveness features. Based on the above, the spliceosome system is becoming an attractive therapeutic target for tumor pathologies [31]. This is the case for pladienolide-B, a natural compound that directly targets and binds a key player in the spliceosome, SF3B1, and thereby inhibits spliceosome functions, which in turns appear to mediate the antitumor properties of this promising drug [31, 32].

To date, the expression pattern and putative role of the core splicing machinery components in the development and progression of PitNETs, as well as the potential therapeutic effects of pladienolide-B in PitNET cells, has not been reported. Accordingly, we aimed to determine and analyze the expression levels of the spliceosome core components and a selected set of relevant SFs in the main PitNETs subtypes, i.e. somatotropinomas (GHomas), non-functioning pituitary tumors (NFPTs), corticotropinomas (ACTHomas) and prolactinomas (PRLomas), as compared to normal human pituitary gland samples. Additionally, we evaluated the potential antitumor actions of pladienolide-B in PitNET cells by evaluating key functional parameters (i.e. cell proliferation/viability and hormone secretion) in human primary PitNETs cell cultures and two pituitary model cell lines (AtT-20 and GH3).

MATERIALS AND METHODS

Drugs and reagents

All reagents and drugs used in this study were purchased from Sigma-Aldrich (Madrid, Spain) or Fluidigm (San Francisco, CA, USA) unless otherwise specified. Pladienolide-B was obtained from Santa Cruz Biotechnology (Heidelberg, Germany).

Patients, samples and primary cell cultures

Human PitNETs samples were collected during transsphenoidal surgery from 171 patients from Spain (90 NFPTs [mean age: 58 (20-83); 39% women], 48 GHomas [mean age: 43 (21-64); 60 % women; Cohort 1; C1], 24 ACTHomas [mean age: 40 (18-61); 78% women], and 9 PRLomas [mean age: 47 (28-74); 38% women]. Moreover, a second cohort of 90 GHomas from Brazil (Cohort 2; C2) was obtained. Additionally, 11 normal pituitary glands (NP) [mean age: 61 (44-85); 50% women] were obtained during autopsies. Each pituitary sample subtype was confirmed by expert anatomo-pathologists and by the molecular screening using quantitative real-time PCR (qPCR), as previously described [17, 33-35]. In all cases, samples were immediately placed in sterile cold medium (S-MEM, Gibco, Madrid, Spain; supplemented with 0.1% BSA, 0.01% L-glutamine, 1% antibiotic-antimycotic solution, and 2,5% HEPES) after surgery and rapidly frozen and stored at -80 ºC until extraction for total RNA. In a second set of experiments, PitNETs samples placed in sterile cold medium after surgery were dispersed into single cells following the methods and reagents previously described [33, 35]. This study was carried out within a project approved by our Hospital Research Ethics Committee, was conducted in accordance with ethical standards of the Helsinki Declaration of the World Medical Association, and written informed consent was obtained from each patient.

Cell lines and culturing

The mouse corticotrope pituitary derived cell line AtT-20/D16v-F2 (ATCC® CRL-1795™) and the rat somatotrope pituitary derived cell line GH3 (ATCC® CCL-82.1™) were used in the

present study. Both cell lines were checked for mycoplasma contamination by PCR [36], cultured in Dulbecco's Modified Eagle's Medium (DMEM) complemented with 10% FBS, 100 U/ml penicillin/streptomycin, 0.024 M of HEPES, and maintained at 37ºC and 5% CO_2, under sterile conditions.

RNA extraction, quantification and reverse transcription

Total RNA from fresh tissue samples was isolated using AllPrep DNA/RNA/Protein Mini Kit followed by DNase treatment using RNase-Free DNase Set (Qiagen; Limburg, Netherlands). Total RNA concentration and purity was assessed using Nanodrop 2000 spectrophotometer (Thermo Fisher; Waltham, MA, Estados Unidos), and retro-transcribed using random hexamer primers with the First Strand Synthesis Kit (Thermo Fisher).

Analysis of splicing machinery components by a customized qPCR dynamic array

As previously described [37, 38], a 48.48 Dynamic Array based on microfluidic technology (Fluidigim) was used to determine the expression levels of 48 transcripts in 48 PitNETs samples, simultaneously. The specific set of primers used in this study has been previously reported by our group [37, 38], and include components of the major (n=13) and minor (n=4) spliceosome, associated SFs (n=28) and three reference genes (ACTB, HPRT1 and GAPDH, used for the normalization of gene expression levels).

We performed a preamplification, exonuclease treatment and the qPCR dynamic array following the manufacturer's instructions. Thus, 12.5ng of cDNA of each sample were pre-amplified using 1µL of PreAmp Master Mix (Fluidigm) and 0.5µL of all primers mix (500nM) in a T100 Thermal-cycler (BioRad, Hercules, CA, USA), using the following program: 1) 2 min at 95 ºC; 2) 15 sec at 94 ºC and 4 min at 60 ºC (14 cycles). Then, samples were treated with 2µL of 4U/µL Exonuclease I solution (New England BioLabs, Ipswich, MA, USA) following manufacturer's instructions. Samples were diluted with 18µL of TE Buffer (Thermo Scientific), and 2.7µL were mixed with 3µL of EvaGreen Supermix (Bio-Rad) and 0.3µL of DNA Binding Dye Sample Loading Reagent (Fluidigm). Primers were diluted to 5µM with 2X Assay Loading

Reagent (Fluidigm). Control line fluid was charged in the chip and Prime script program was run into the IFC controller MX (Fluidigm). Finally, 5μL of each primer and 5μL of each sample were pipetted into their respective inlets on the chip and the Load Mix script in the IFC controller software was run. After this program, the qPCR was run using Biomark System (Fluidigm) with the following thermal profile: 1) 1 min at 95 ºC; 2) 35 cycles of denaturing (5 sec at 95 ºC) and annealing/extension (20 sec at 60 ºC); and 3) a last cycle where final products were subjected to graded-temperature-dependent dissociation (60 ºC to 95 ºC, increasing 1ºC/3 sec). Results were processed with Real-Time PCR Analysis Software 3.0 (Fluidigm).

Measurement of cell proliferation/viability

As previously reported [17, 33, 34], 10,000 cells per well (for PitNET cells) and 6,000 cells per well (for cell lines) were plated in 96-well plates to measure cell proliferation/viability every 24h until 72h using Alamar-blue reagent (Invitrogen, Madrid, Spain). Pladienolide-B was daily refreshed after each measurement, and cell proliferation/viability was evaluated using Flex-Station III System (Molecular Devices, Sunnyvale, CA, USA).

Measurement of hormone secretion

We plated 150,000-200,000 cells per well in 24-well plates in serum-containing media. GH-secreting PitNETs cells were used to analyze the effect of pladienolide-B on GH secretion after 24h of incubation in serum-free media. GH was measured using human commercial ELISA kit (reference number: EIA-3552; DRG, Mountainside, NJ), according to the manufacturer's instructions.

Statistical analysis

All data were evaluated for heterogeneity of variance using the Kolmogorov-Smirnov test. Statistical differences from qPCR dynamic array results were evaluated by unpaired nonparametric Mann Whitney test and data were expressed as mean ± interquartile range. As previously reported [18, 30], ROC curves were used as a tool to measure how well the

202 expression of splicing machinery components could discriminate between different diagnostic
203 groups. Statistical analysis of ROC curves was performed by calculating the Area Under the
204 Curve (AUC) of each component and comparing them with the AUC of the reference line using
205 Student's t-test. Heatmaps and clustering analysis were performed using MetaboAnalyst 3.0
206 [39]. Statistical analyses from functional assays were assessed by paired parametric t-test or
207 one-way ANOVA test followed by Dunnett's test for multiple comparisons, and data were
208 expressed as mean ± SEM. As previously reported, to normalize values within each treatment
209 and minimize intragroup variations in the different *in vitro* experiments (i.e., different age of the
210 tissue donor or metabolic environment), the values obtained were compared with vehicle-treated
211 controls (set at 100%). All experiments were performed in a minimum of three different primary
212 pituitary cultures from different patients (three or four replicates per treatment per experiment),
213 unless otherwise specified. P values ≤ 0.05 were considered statistically significant. A trend for
214 significance was indicated when P values ranged between >0.05 and <0.1. All statistical
215 analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA).
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RESULTS

Dysregulation of splicing machinery in NFPTs

Results from a dynamic qPCR microfluidic custom-made array revealed a marked dysregulation of the expression levels of several components of the splicing machinery in NFPTs compared to NPs, wherein most elements were clearly downregulated (20 out of 44) or exhibited a trend to be downregulated in NFPTs (Figure-1A; Supplemental Figure-S1). Specifically, NFPTs showed a significant downregulation of 3 major spliceosome components (RNU6, U2AF1 and U2AF2), 2 minor spliceosome components (RNU11 and RNU6ATAC), and 15 SFs (CELF4, CELF1, ESRP1, SRRM4, PTBP1, RBM17, RBM45, SND1, SRSF1, SRSF10, SRSF3, SRSF5, SRSF9, TRA2A, and TRA2B) (Figure-1A and Supplemental Figure-S1). Moreover, although non-supervised hierarchical analysis based on the expression pattern of all spliceosome components and SFs analyzed was not able to appropriately separate NFPTs from NPs (Figure-1B), Partial Least Squares-Discriminant (PLS-DA) analysis suggested the possibility of discriminating between NFPTs and NPs using the expression pattern of spliceosome components and SFs (Figure-1C). Indeed, Variable Importance in Projection (VIP) score of PLS-DA analysis indicated that SRSF9, SND1, U2AF1 and CELF4 were the components with higher capacity to discriminate between both populations (Figure-1D). In fact, although the heatmap generated with the expression of these four components did not produce a complete clustering of NFPTs and NPs, it was able to cluster together all NP-samples independently of NFPT samples. Moreover, ROC curve analyses of these 4 components corroborated their capacity to discriminate between NFPTs and NPs showing an AUC of 0.94, 0.94, 0.93 and 0.89, respectively ($p<0.001$; Figure-1F).

Dysregulation of splicing machinery in GHomas

In GH-secreting PitNETs, a clear dysregulation of splicing machinery components was also found compared to NPs (expression of 24 out of 44 elements were significantly altered; Figure-2A and supplemental Figure-S2). Specifically, analysis of cohort-1 of GHomas (tumors from

Spain) showed an overexpression of 6 major spliceosome components (SNRNP200, U2AF1, U2AF2, TCERG1, PRPF8 and RBM22), a downregulation of one component of the minor spliceosome (RNU11), and 17 significantly altered SFs [16 upregulated (CELF4, CELF1, MAGOH, SRRM4, SPFQ, PTBP1, RAVER1, RBM17, RBM3, KHDRSB1, SRSF2, SND1, SRRM1, SRSF3, SRSF5, SRSF6, SRSF9, TIA1 and TRA2B) and one downregulated (ESRP2)] (Figure-2A and supplemental Figure-S2). A non-supervised hierarchical analysis with the expression levels of all the splicing machinery components was able to cluster together all NP-samples independently of GHoma samples, but did not generate a complete clustering of GHomas and NPs (Figure-2B). However, PLS-DA analysis showed a clear segregation between GHomas and NPs (Figure-2C) and further analysis revealed that the pattern of three SFs with the highest score in the VIP analysis (RAVER1, RBM3 and SRSF6; Figure-2D) was able to discriminate between GHomas and NPs in two perfect clusters (Figure-2E). Moreover, ROC curve analyses of these three SFs (RAVER1, RBM3, and SRSF6) showed an AUC of 0.99, 1, and 0.98, respectively (Figure-2F). Additionally, we had the opportunity to corroborate these results in another cohort of GHomas from Brazil (cohort-2; C2). In this case, we confirmed the overexpression of RAVER1 and RBM3, but not SRSF6, in this cohort of GHomas compared to NPs. Moreover, ROC curves analyses of RAVER1 and RBM3, but not of SRSF6, confirmed their capacity to discriminate between NPs and the validation cohort (C2; Figure-2F) with an AUC of 0.77, 0.93, and 0.59, respectively.

Dysregulation of splicing machinery in ACTHomas

In ACTH-secreting PitNETs, qPCR array revealed a significantly dysregulation of 11 splicing machinery components, two components of major spliceosome (upregulation of U2 and downregulation of U2AF2) and 9 altered SFs (upregulation of CELF4, MAGOH, NOVA1, SPFQ, KHDRSB1, SRSF2, SNW1 and TRA2B, and downregulation of ESRP1) (Figure-3A and supplemental Figure-S3). Non-supervised hierarchical analysis of all splicing machinery components analyzed did not generate a clustering able to discriminate between ACTHomas and NPs (Figure-3B) but PLS-DA analysis showed a clear separation between ACTHomas and

NPs (Figure-3C). Further analysis revealed that the pattern of two SFs with the highest score in the VIP analysis (MAGOH and KHDRSB1; Figure-3D) was able to discriminate between ACTHomas and NPs in two perfect clusters (Figure-3E). Moreover, ROC curve analyses of MAGOH and KHDRSB1 corroborated their capacity to discriminate between ACTHomas and NPs with an AUC of 1 and 0.97, respectively (Figure-3F).

Dysregulation of splicing machinery in PRLomas

PRLomas also exhibited a clearly dysregulated expression pattern of spliceosomal components (18 out of 44) compared to NPs (Figure-4A and supplemental Figure-S4), with a significant overexpression of 3 major spliceosome components (PRPF40A, PRPF8, and RBM22), a downregulation of one minor spliceosome components (RNU11), and an alteration of 14 SFs [12 upregulated (CELF1, MAGOH, SRRM4, PTBP1, RAVER1, RBM3, KHDRSB1, SRSF2, SNW1, SRSF3, SRSF6, and TIA1) and 2 downregulated (ESRP2 and SRSF1)] (Figure-4A and supplemental Figure-S4). Although the non-supervised hierarchical analysis did not identify a perfect clustering between PRLomas and NPs, both populations were visually well differentiated in the heatmap (Figure-4B). However, although PLS-DA analysis suggested a different expression pattern between PRLomas and NPs (Figure-4C) and VIP analysis revealed several components with high capacity to discriminate between both populations (Figure-4D), any combination of these high-scored factors was able to improve the clustering to discriminate between PRLomas and NPs (Figure-4E). The best combination included minor spliceosome components and SFs (RNU11, ESRP2, RNU6ATAC, SRSF1, and ESRP1) with an AUC of 0.96, 0.91, 0.73, 0.76, and 0.74, respectively (Figure-4F).

Similar dysregulation of specific splicing machinery components in all PitNET subtypes.

A fold-change representation of the splicing machinery alterations in all PitNETs subtypes analyzed revealed a common fingerprint between all of them (Supplemental Figure-S5A). Specifically, we found a common downregulation of three minor spliceosome components

(RNU11, RNU4ATAC and RNU6ATAC) and one SF (SRSF1). However, these changes did not reach statistically significance in all of them (Supplemental Figure-S5B).

Effect of Pladienolide-B treatment in PitNETs cells.

Dose-response experiments using pladienolide-B in pituitary cell lines, corticotrope AtT-20 and somatotrope GH3, at different times of incubation showed that lower doses of pladienolide-B (10^{-9} and 10^{-11} M) did not alter cell proliferation at any of the times tested (Figure-5A). In contrast, 10^{-7}M pladienolide-B markedly decreased cell proliferation at 24, 48 and 72h in both cell lines (Figure-5A). Based on these results, the 10^{-7}M dose of pladienolide-B was used in NFPT, GHoma and ACTHoma cell cultures, which revealed that cell viability was clearly reduced after 72h of incubation in NFPTs, and after 48 and 72h of incubation in GHomas cell cultures. In ACTHomas, results revealed a clear numerical decrease of cell viability after 72h of incubation, but these results could not be analyzed statistically, due to the limited number of samples available to be tested with this PitNET subtype (n=2). Finally, treatment with pladienolide-B significantly reduced GH secretion after 24h of incubation in GHomas.

DISCUSSION

Evidence gathered over the last years indicates that tumor pathologies, including NETs share as a common feature the altered expression of functionally and pathologically relevant splicing variants of diverse molecules, from membrane receptors to key signaling enzymes (DLK1, GHRHR, IGF1R, EGFR, CSH2 or PTEN) [40-44]. Actually, results from our group led to the identification of previously unrecognized aberrant splicing variants from somatostatin and ghrelin systems (SST_5TMD4/5 and In1-ghrelin), and demonstrated that these variants are overexpressed in tumors and can contribute to their oncogenesis, increasing aggressiveness and malignant features in different tumor types, including PitNETs [17-22, 30, 45, 46]. To ascertain the potential mechanisms underlying the genesis of these tumor-related abnormal splicing events, we hypothesized that they could be linked to alterations in the machinery responsible for this process, i.e. the spliceosome core and its associated SFs. In line with this notion, mutations and other functional defects in certain spliceosome components have been reported to cause diverse pathologies, including cancer [47]. Accordingly, the present study was devised to determine the pattern of expression of the splicing machinery in the main types of PitNETs and to assess the potential existence of specific alterations in spliceosome components and SFs associated to each type of tumor, which may serve as future tools to guide the diagnostic/prognostic of these tumors, and could provide novel actionable therapeutic targets. Indeed, results from this study demonstrate, for first time, that the splicing machinery (spliceosome and SFs) is distinctly dysregulated in PitNETs compared to NP glands, and that its modulation with a specific drug targeting SF3b1, a key player in the spliceosome function, decreases aggressiveness features in PitNET cell cultures.

One of the main findings of this study is the discovery that the spliceosome machinery is dysregulated in a tumor subtype-dependent manner, where NFPTs, GHomas, ACTHomas and PRLomas exhibit a differentially altered pattern of expression. Of particular interest are the results found in NFPTs, which displayed a profound downregulation of most of the components analyzed, in striking contrast with the alterations observed in functioning PitNETs (GHomas, ACTHomas and PRLomas). In line with this, previous results have demonstrated that NFPTs

have a dissimilar behavior and different expression pattern of relevant components involved in pituitary cell function, such as somatostatin receptors, in comparison with functioning PitNETs and normal tissue [33, 48-50]. Interestingly, our results showed that the expression levels of SRSF9, SND1, U2AF1 and CELF4 were able to discriminate, although not perfectly, between NFPTs and NP tissues. The absence of a perfect discrimination between both populations could be due to the intrinsic heterogeneous nature of NFPTs [51]. Nonetheless, a clear alteration of these spliceosome components found in our cohort of NFPTs has also been observed in other tumor pathologies. Specifically, SRSF9 and SND1 have been found overexpressed in several tumor pathologies such as breast cancer, bladder cancer, glioblastoma, melanoma or hepatocellular carcinoma, where they have been related with an increase in cell proliferation, invasion and poor prognosis [52-56]. Moreover, CELF proteins have been reported to target and regulate the splicing of neurofibromatosis type 1 (NF1) protein generating a protein with the exon 23a excluded, which has 10 times greater ability to regulate Ras signaling, a main component of MAPK signaling pathway [57]. In addition, U2AF1 is an important component of the major spliceosome that has been found frequently mutated and associated to the generation of particular splicing patterns in several pathologies, including the production of oncogenic splicing variants in cancer [58, 59]. In this sense, our data showed a clear downregulation of U2AF1 in NFPTs compared to NPs, which might suggest that not only the mutation pattern but also the expression pattern could be involved in the malignant behavior of tumor pathologies including NFPTs.

In a first cohort of GHomas, we found a profound overexpression of three SFs, RAVER1, RBM3 and SRSF6, whose expression pattern clearly discriminated between GHomas and NPs. Importantly, the altered expression pattern of RAVER1 and RBM3 was corroborated in a second, independent cohort of GHomas. Interestingly, previous results from our group have revealed that the alteration of these spliceosome components could be associated to the development of different pathological conditions. Indeed, the dysregulation of RAVER1 and RBM3 has been related with the development of non-alcoholic fatty liver disease [38], while RAVER1 has been found to be dysregulated in patients with cardiovascular disease at higher

risk of type-2 diabetes development [37]. But most importantly, additional evidence suggests that alterations in the expression level of RBM3 could be associated with advanced pathological tumor stages in lung carcinoma or with aggressive features in esophageal, colorectal or breast cancer [60-63], which reinforces the crucial role of this factor in tumor pathologies. Moreover, our results with SRSF6 in the first cohort analyzed are also in accordance with the changes reported in colorectal cancer, where SRSF6 was associated with poor prognosis and was postulated as a possible therapeutic target to reduce tumorigenesis [64].

In ACTHomas, our results demonstrated that only the altered expression of two SFs, MAGOH and KHDRSB1, was sufficient to clearly discriminate between ACTHomas and NPs. These SFs were significantly upregulated in ACTHomas, which is in accordance with the increased expression of KHDRSB1 found in gastric cancer, epithelial ovarian cancer or sacral chordomas, wherein its presence was associated with poor prognosis and aggressive characteristics [65, 66]. Likewise, MAGOH has been shown to be differentially expressed in breast cancer, where it served, together with other RNA processing factors, to develop a robust stratification of breast cancer subtypes [67]. However, the presence and potential role of KHDRSB1 and MAGOH in PitNETs or normal pituitary has not been reported hitherto.

Our data in PRLomas revealed that only the combination of all spliceosome components was able to distinguish, although not in a perfect manner, between PRLomas and NPs. Conversely, the combination of the components with higher score in VIP analysis was not sufficient to distinguish between both populations. This might probably be associated to the low number of PRLomas analyzed in this study, owing to the difficulty to have access to this type of samples since dopamine agonists treatment are highly successful in patients with PRLomas.

Together with the identification of clearly distinct, tumor type-dependent dysregulations of the components of the splicing machinery, it is worth noting that we also pinpointed a common downregulation of three minor spliceosome components (RNU11, RNU4ATAC and RNU6ATAC) and one SF (SRSF1) in most PitNETs, irrespective of their subtype, an observation which might be patho-physiologically relevant. In particular, SRSF1 has been described to interact with many different proteins to regulate several cellular functions,

including of course splicing, and has been found overexpressed in several types of cancer (breast and lung cancer), where it is considered a proto-oncogene [68]. The fact that these spliceosome components are similarly dysregulated in all PitNETs, despite the high heterogeneity of these tumors, invite to speculate about the existence of common driver alterations in pituitary tumorigenesis, which would pave the way toward the identification of common therapeutic targets based on the dysregulations of these key elements. However, further studies should be conducted to test this hypothesis.

Finally, our study also provides an initial unprecedented proof-of-concept on the suitability of splicing dysregulation as a novel potential target for PitNET treatment, by demonstrating that the pharmacological disruption of the splicing process with specific drugs may have antitumor effects in these neoplasms. In particular, we tested the direct effect of pladienolide-B in different PitNETs subtypes and pituitary cell lines. This compound is able to directly target a key component involved in the assembly of the spliceosome SF3B1 [69], leading to the reduction of its activity [70]. Several reports have associated pladienolide-B with antitumor properties in different cancer types [32, 71-73], but its role in PitNETs was still unknown. Our results demonstrate for the first time that treatment with pladienolide-B inhibits cell viability/ proliferation in all PitNETs subtypes tested and in AtT-20 and GH3 cell lines, which is in line with the reduction on cell viability and colony formation observed in *HeLa* cells [32]. NFPTs were less sensitive to the effect of pladienolide-B compared to GHomas or ACTHomas, which is in line with previous observations in response to other treatments in NFPTs [33, 48, 74]. Notably, pladienolide-B was also able to reduce GH secretion after 24h of incubation, a relevant result since tumor hypersecretion is linked to most of the symptoms caused by GHomas.

In summary, the present results provide novel, compelling evidence to propose that the splicing machinery is severely and distinctly dysregulated in the main subtypes of PitNETs compared to NPs, and identified unique fingerprints of spliceosome components in each PitNETs subtype that can accurately discriminate between normal and tumor pituitary tissues. Furthermore, we also found several components, including SFs (SRSF1) and specially three minor spliceosome components (RNU11, RNU4ATAC and RNU6ATAC), commonly

dysregulated in all PitNET subtypes, which could represent novel, common therapeutic targets in these pathologies. These discoveries open a new window to investigate the plausible contribution of splicing dysregulation and its subsequent outcomes to pituitary tumorigenesis, and to assess the potential value of specific splicing machinery components as novel diagnostic/prognostic tools in these pathologies. Furthermore, our study unveils splicing, particularly SF3b1, as a novel actionable therapeutic point that can be targeted by Pladienolide-B to combat PitNETs.

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636 **FIGURE CAPTIONS**

637

638 **Figure 1:** Non-functioning pituitary tumors (NFPTs). (A) Heatmap of the mRNA expression
639 levels of all spliceosome components measured in the qPCR array in NFPTs (n=90; green color)
640 compared to NPs (n=11; red color). (B) Individual Fold-Change of each spliceosome
641 component expression levels in NFPTs compared to NPs. (C) Principal Components Analysis
642 (PCA) of the mRNA expression levels of the spliceosome components analyzed in the same set
643 of samples. (D) Vip Scores top-feature of Partial Least Squares Discriminant Analysis (PLS-
644 DA). (E) Heatmap of the spliceosome components with higher vip score in the same set of
645 samples. (F) mRNA expression levels of spliceosome components with higher vip score in
646 NFPTs compared to NPs and ROC curves analyses showing the accuracy of the selected
647 spliceosome components to discriminate between NFPTs and NPs. Data represent median ±
648 interquartile range of absolute expression levels (copy number) of each transcript adjusted by a
649 normalization factor. Asterisks (*** $p<0.001$) indicate statistically significant differences
650 between groups.

651

652 **Figure 2:** GH-secreting PitNETs (A) (A) Heatmap of the mRNA expression levels of all
653 spliceosome components measured in the qPCR array in GHomas (n=48; green color) compared
654 to NPs (n=11; red color). (B) Individual Fold-Change of each spliceosome component
655 expression levels in GHomas compared to NPs. (C) Principal Components Analysis (PCA) of
656 the mRNA expression levels of the spliceosome components analyzed in the same set of
657 samples. (D) Vip Scores top-feature of Partial Least Squares Discriminant Analysis (PLS-DA).
658 (E) Heatmap of the spliceosome components with higher vip score in the same set of samples.
659 (F) mRNA expression levels of spliceosome components with higher vip score in GHomas from
660 cohorts 1 (C1; n=48) and 2 (C2; n=96) compared to NPs (n=11) and ROC curves analyses
661 showing the accuracy of the selected spliceosome components to discriminate between both
662 cohorts of GHomas and NPs. Data represent median ± interquartile range of absolute expression

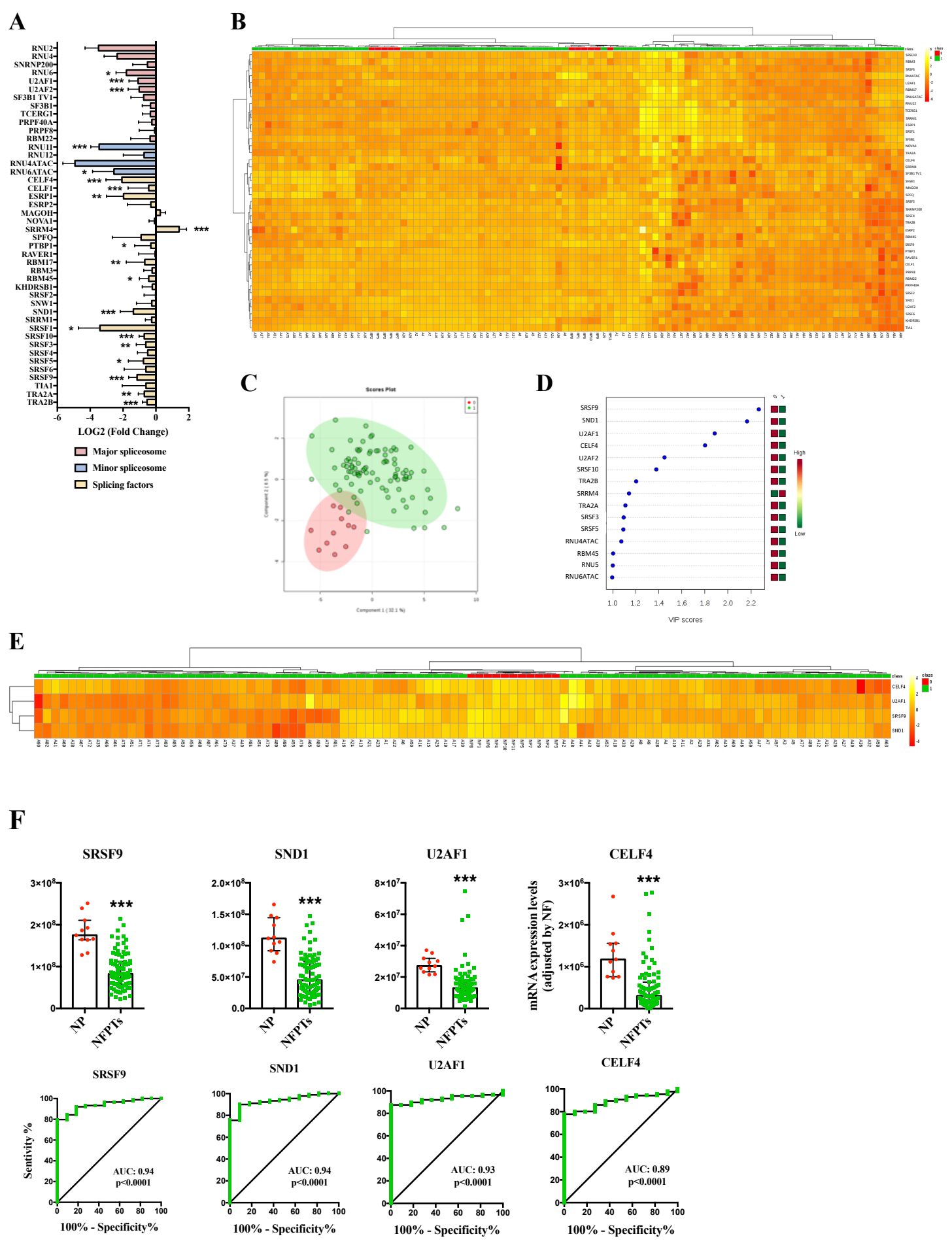
levels (copy number) of each transcript adjusted by a normalization factor. Asterisks (** $p<0.01$, *** $p<0.001$) indicate statistically significant differences between groups.

Figure 3: ACTH-secreting PitNETs. (A) Heatmap of the mRNA expression levels of all spliceosome components measured in the qPCR array in ACTHomas (n=24; green color) compared to NPs (n=10; red color). (B) Individual Fold-Change of each spliceosome component expression levels in ACTHomas compared to NPs. (C) Principal Components Analysis (PCA) of the mRNA expression levels of the spliceosome components analyzed in the same set of samples. (D) Vip Scores top-feature of Partial Least Squares Discriminant Analysis (PLS-DA). (E) Heatmap of the spliceosome components with higher vip score in the same set of samples. (F) mRNA expression levels of spliceosome components with higher vip score in ACTHomas compared to NPs and ROC curves analyses showing the accuracy of the selected spliceosome components to discriminate between ACTHomas and NPs. Data represent median ± interquartile range of absolute expression levels (copy number) of each transcript adjusted by a normalization factor. Asterisks (*** $p<0.001$) indicate statistically significant differences between groups.

Figure 4: PRL-secreting PitNETs. (A) Heatmap of the mRNA expression levels of all spliceosome components measured in the qPCR array in PRLomas (n=9; green color) compared to NPs (n=11; red color). (B) Individual Fold-Change of each spliceosome component expression levels in PRLomas compared to NPs. (C) Principal Components Analysis (PCA) of the mRNA expression levels of the spliceosome components analyzed in the same set of samples. (D) Vip Scores top-feature of Partial Least Squares Discriminant Analysis (PLS-DA). (E) Heatmap of the spliceosome components with higher vip score in the same set of samples. (F) mRNA expression levels of spliceosome components with higher vip score in PRLomas compared to NPs and ROC curves analyses showing the accuracy of the selected spliceosome components to discriminate between PRLomas and NPs. Data represent median ± interquartile range of absolute expression levels (copy number) of each transcript adjusted by a

normalization factor. Asterisks (* $p<0.05$; *** $p<0.001$) indicate statistically significant differences between groups.

Figure 5: Functional assays in response to pladienolide-B in pituitary cell lines and PitNETs primary cell cultures. (A) Dose-response experiments of cell proliferation in response to pladienolide-B at 10^{-7}, 10^{-9}, and 10^{-11} M in GH3 and AtT20 cells (n=4), measured by Alamar-blue reduction. (B) Dose-response experiments of cell viability in response to pladienolide-B in NFPTs (n=5), GHomas (n=3), and ACTHomas (n=2), measured by Alamar-blue reduction. (C) Effect of pladienolide-B in GH secretion in GHomas (n=2), determined by commercial ELISA kit. Data are expressed as percent of vehicle-treated controls (set at 100%) within experiment. Values represent the mean ± SEM. Asterisks (* $p<0.05$; ** $p<0.01$; *** $p<0.001$) indicate statistically significant differences.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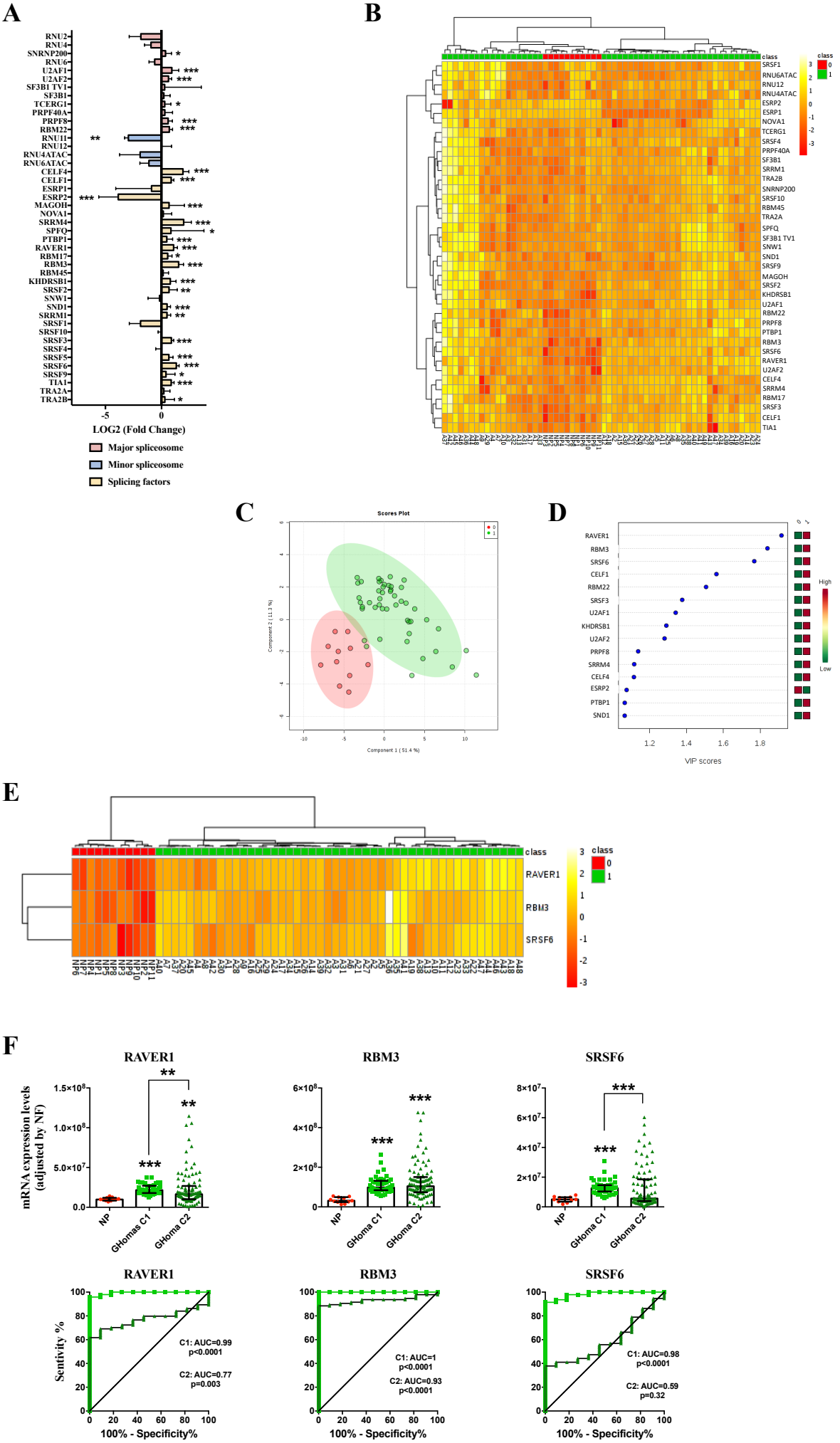
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mRNA expression levels (adjusted by NF)
NP
GHomas C1
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100% - Specificity%
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p<0.0001
C2: AUC=0.77
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C1: AUC=1
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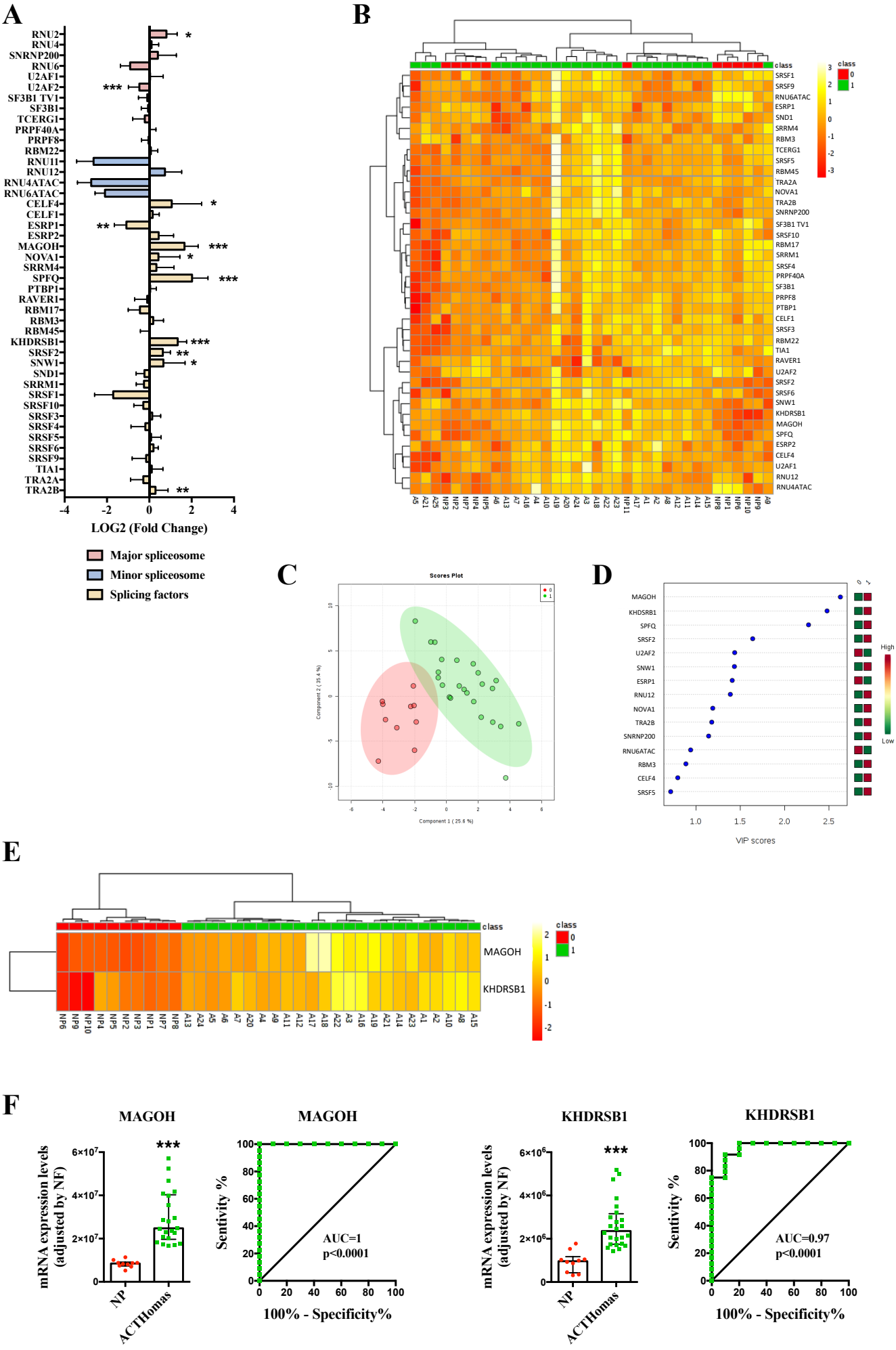
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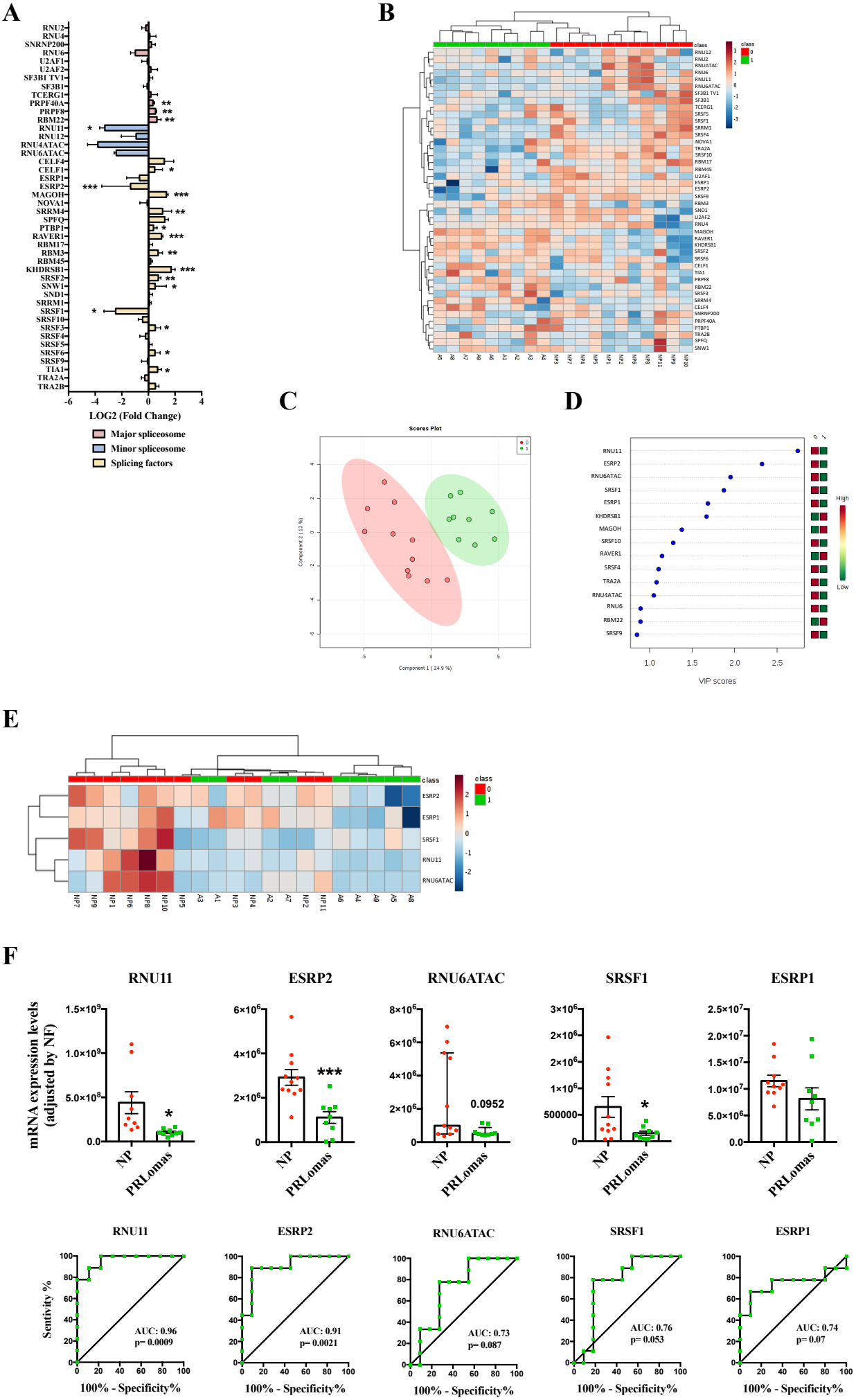
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Splicing factors
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Component 1 (24.9 %)
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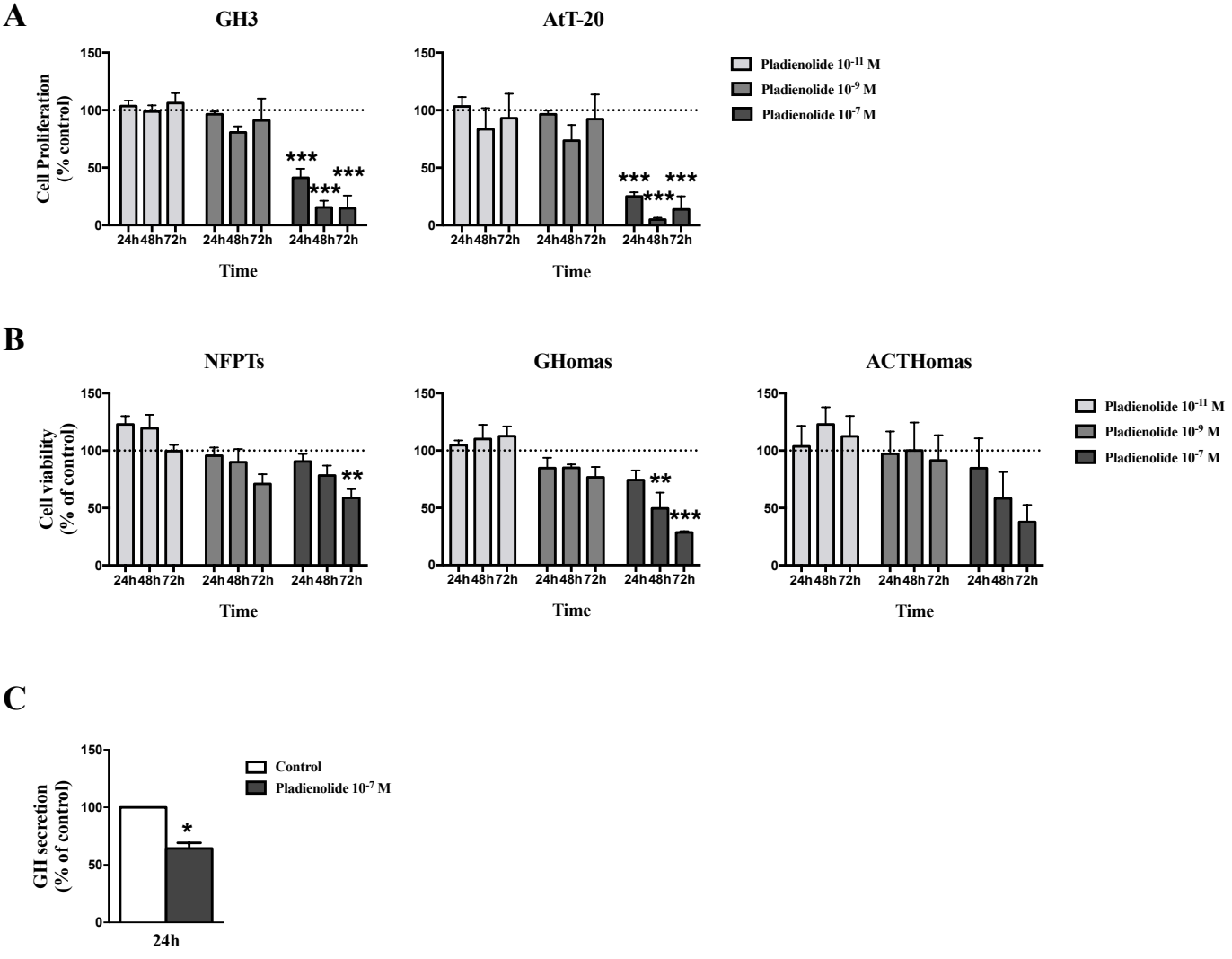
RNU11
ESRP2
RNU6ATAC
SRSF1
ESRP1
mRNA expression levels (adjusted by NF)
NP
PRLomas
0.0952
Sentivity %
100% - Specificity%
AUC: 0.96
p= 0.0009
AUC: 0.91
p= 0.0021
AUC: 0.73
p= 0.087
AUC: 0.76
p= 0.053
AUC: 0.74
p= 0.07


A
GH3
AtT-20
Cell Proliferation (% control)
150
100
50
0
***
***
***
24h 48h 72h
Time
Pladienolide 10-11 M
Pladienolide 10-9 M
Pladienolide 10-7 M
B
NFPTs
GHomas
ACTHomas
Cell viability (% of control)
**
**
***
C
GH secretion (% of control)
Control
Pladienolide 10-7 M
*
24h

Supplementary Information

Splicing machinery is dysregulated in pituitary neuroendocrine tumors (PitNETs) and associated with aggressiveness features

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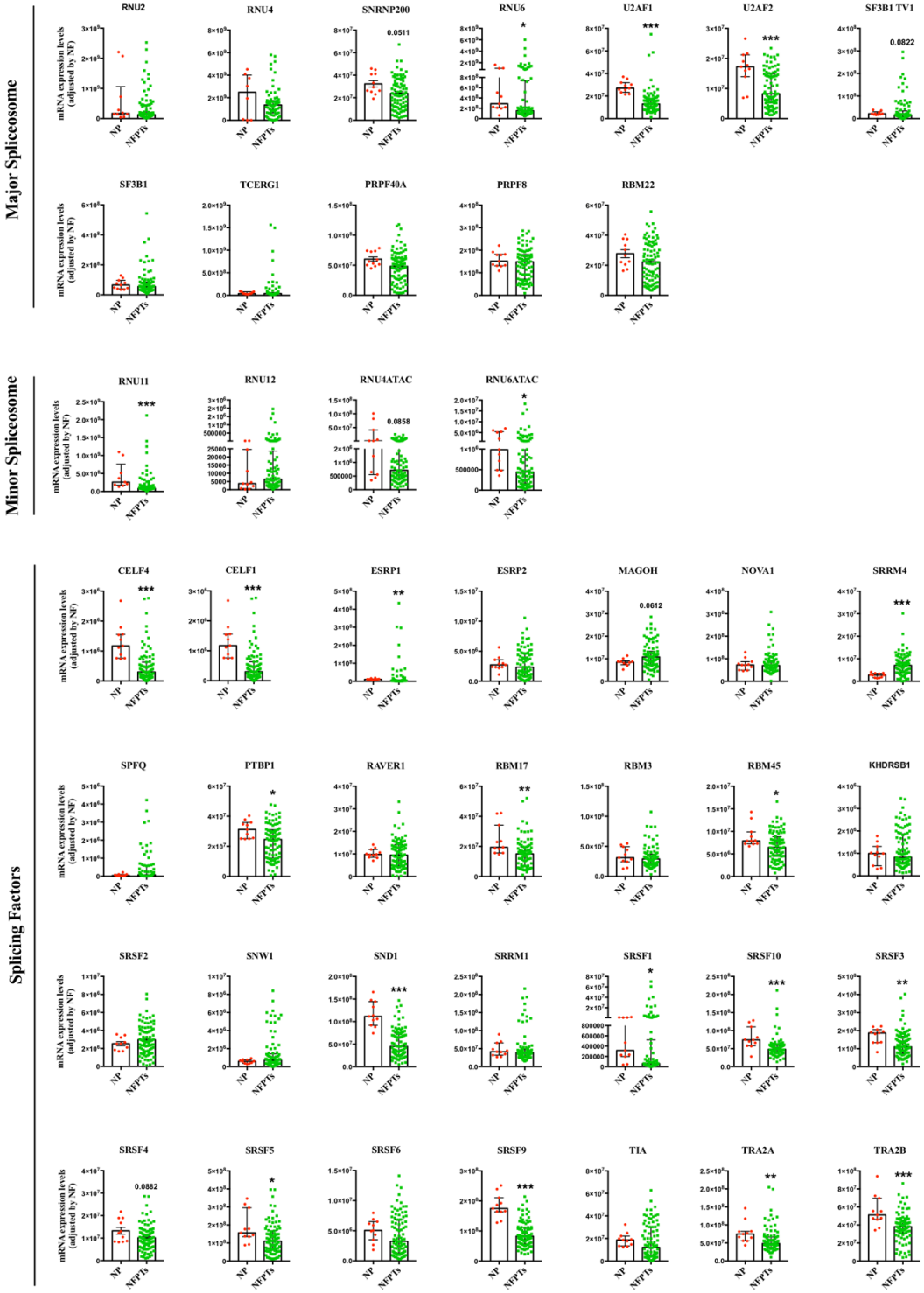
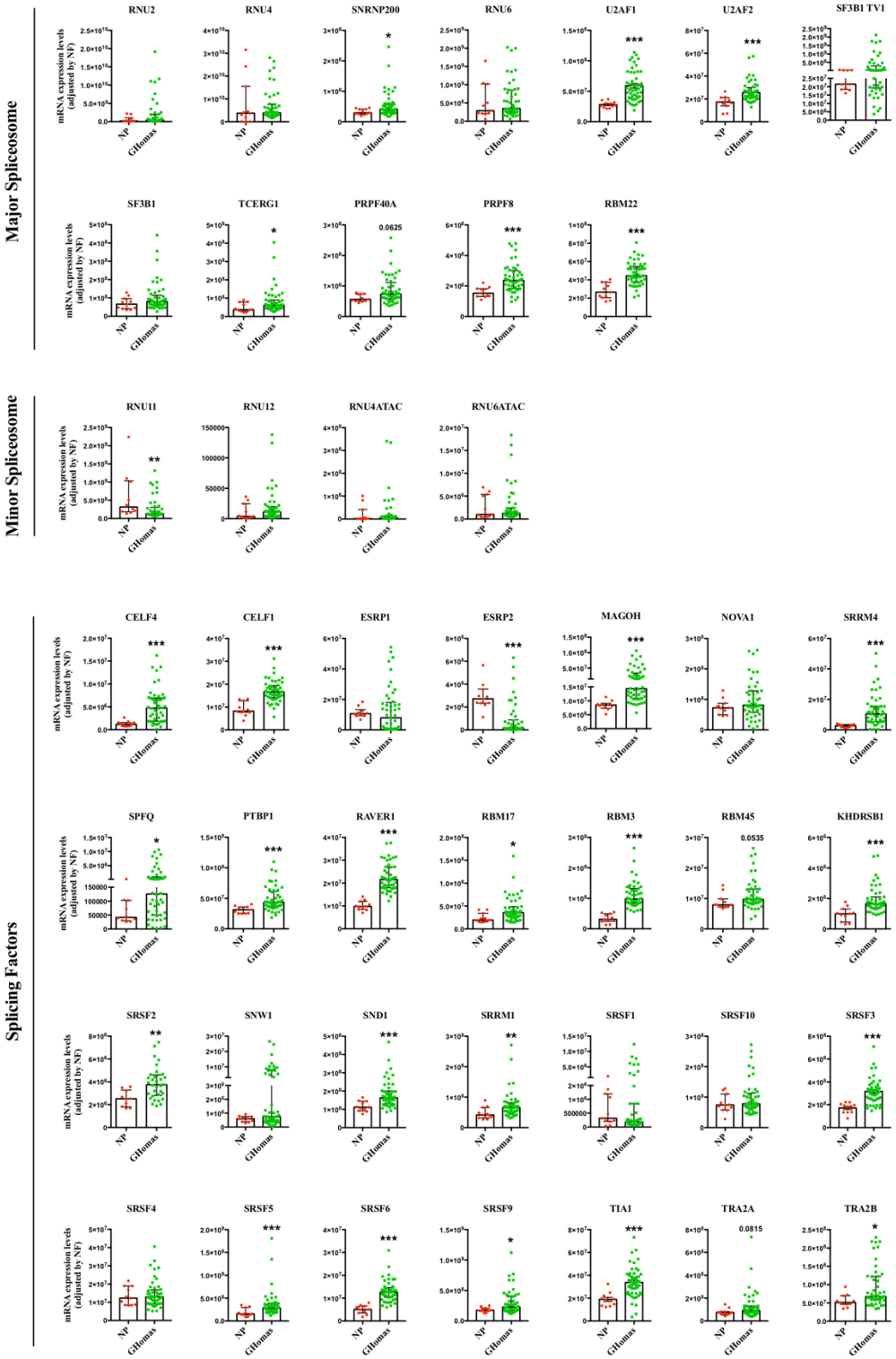
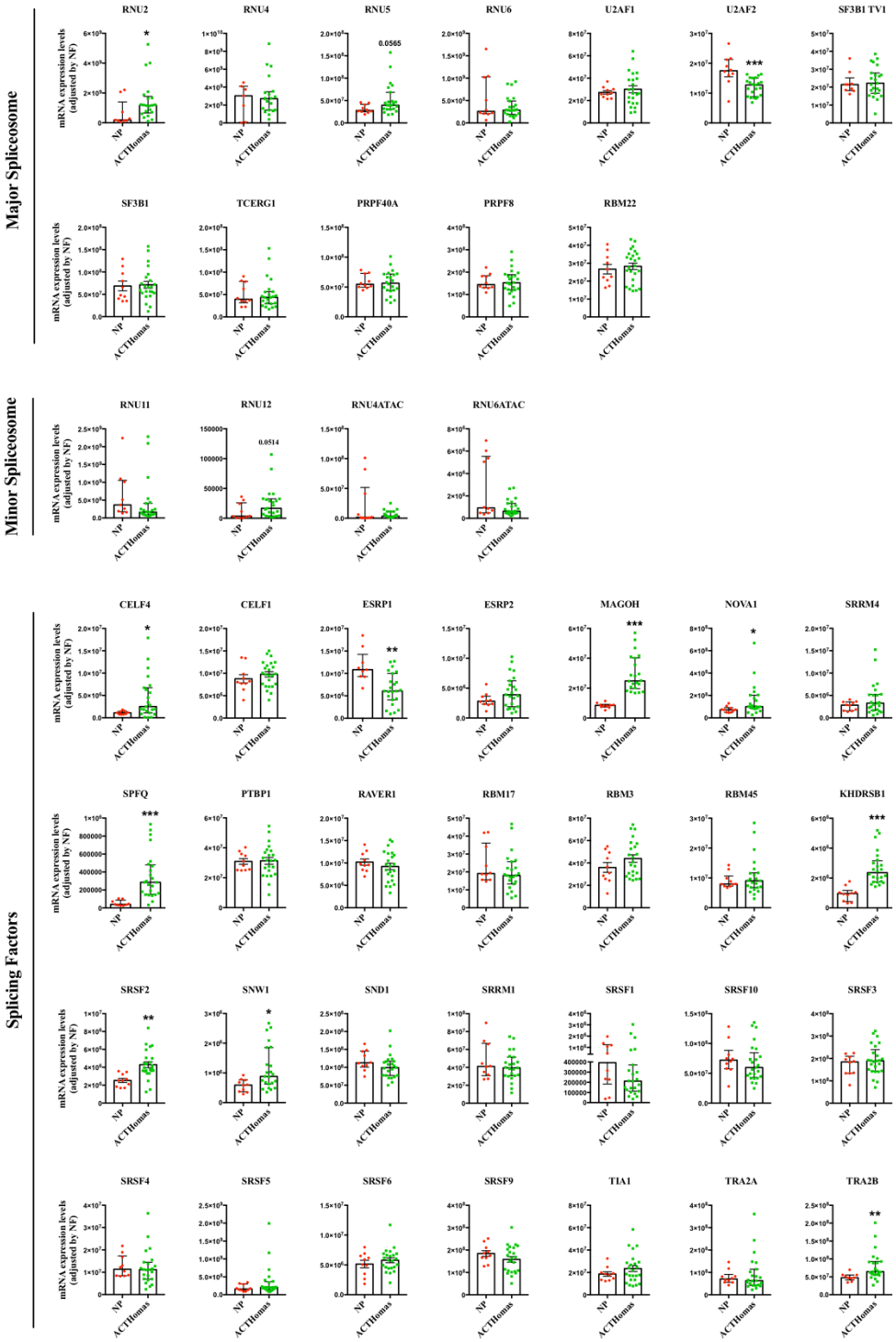
Supplemental Figure 1: Non-functioning pituitary tumors (NFPTs). mRNA expression levels of all spliceosome components and splicing factors measured in NFPTs compared to NPs using the qPCR array. Data represent median ± interquartile range of absolute expression levels (copy number) of each transcript adjusted by a normalization factor. Asterisks (* p<0.05, ** p<0.01, *** p<0.001) indicate statistically significant differences between groups.



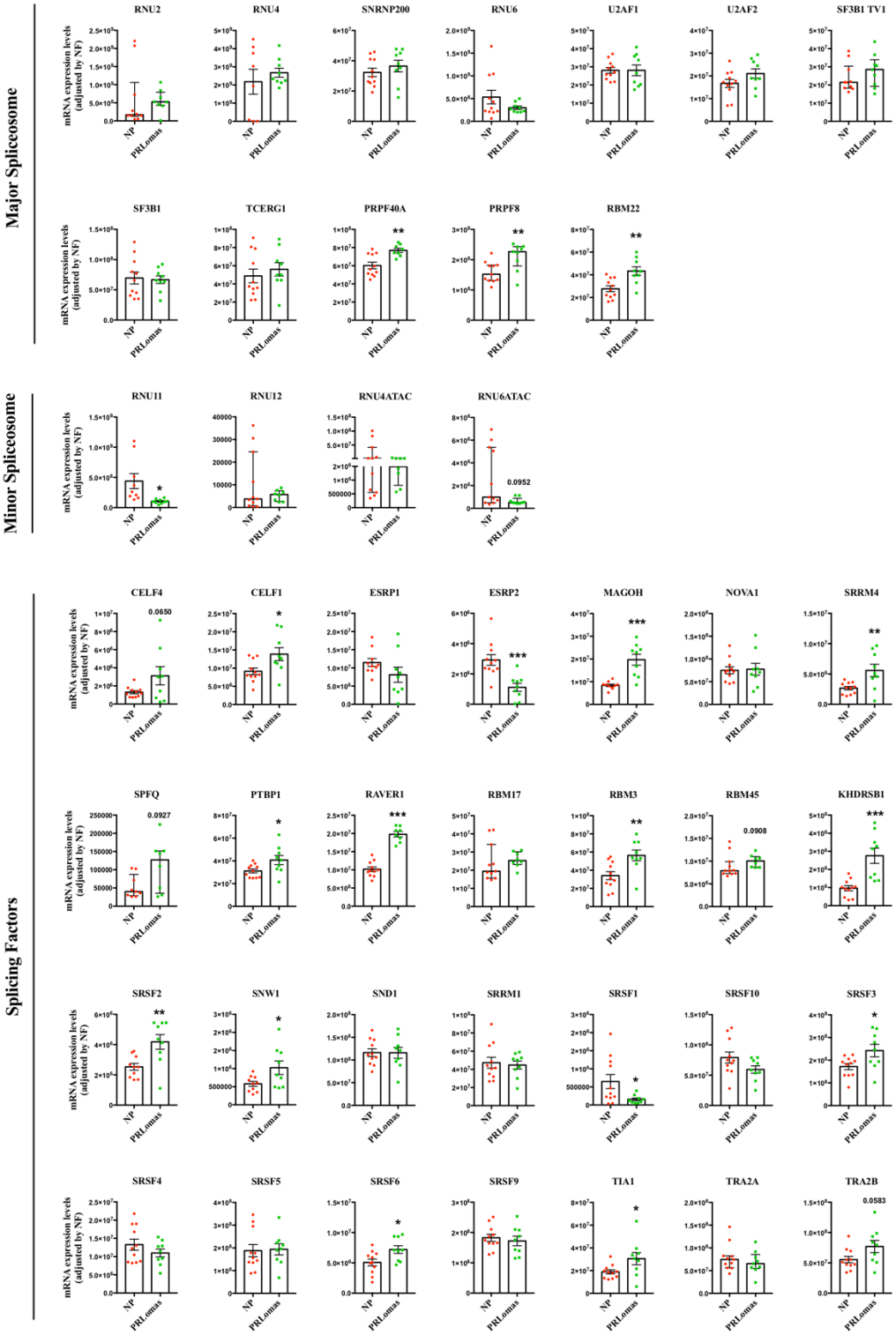
Supplemental Figure 2: GH-secreting PitNETs (GHomas). mRNA expression levels of all spliceosome components and splicing factors measured in GHomas compared to NPs using the qPCR array. Data represent median ± interquartile range of absolute expression levels (copy number) of each transcript adjusted by a normalization factor. Asterisks (* p<0.05, ** p<0.01, *** p<0.001) indicate statistically significant differences between groups.



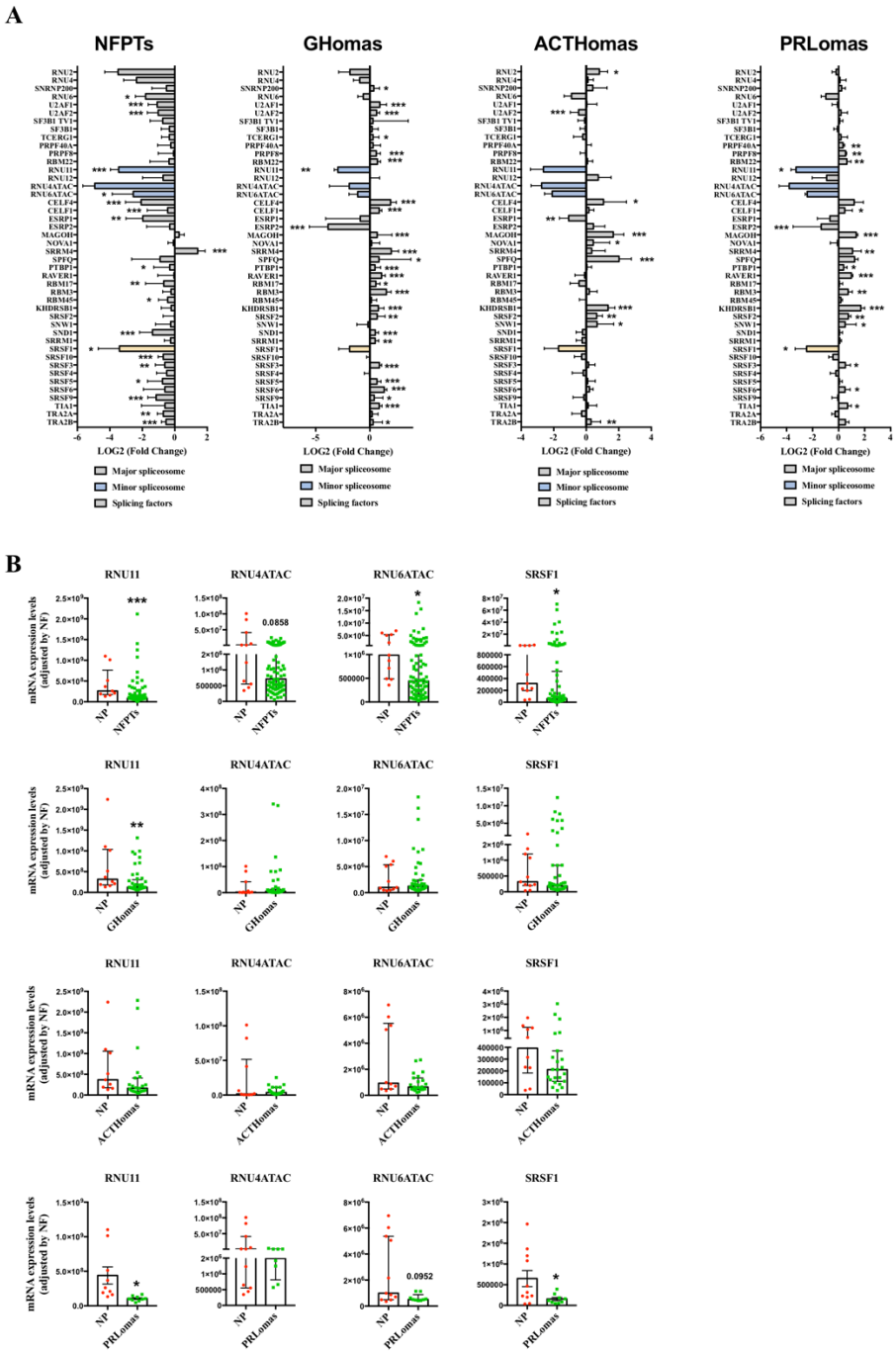
Supplemental Figure 3: ACTH-secreting PitNETs (ACTHomas). mRNA expression levels of all spliceosome components and splicing factors measured in ACTHomas compared to NPs using the qPCR array. Data represent median ± interquartile range of absolute expression levels (copy number) of each transcript adjusted by a normalization factor. Asterisks (* p<0.05, ** p<0.01, *** p<0.001) indicate statistically significant differences between groups.



Supplemental Figure 4: PRL-secreting PitNETs (PRLomas). mRNA expression levels of all spliceosome components and splicing factors measured in PRLomas compared to NPs using the qPCR array. Data represent median ± interquartile range of absolute expression levels (copy number) of each transcript adjusted by a normalization factor. Asterisks (* p<0.05, ** p<0.01, *** p<0.001) indicate statistically significant differences between groups.



Supplemental Figure 5: (A) Individual Fold-Change of spliceosome machinery expression levels showing the common dysregulated components (blue and yellow colors) in all PitNETs subtypes compared to NPs. (B) mRNA expression levels of spliceosome components and the splicing factor commonly dysregulated in all PitNETs. Data represent median ± interquartile range of absolute expression levels (copy number) of each transcript adjusted by a normalization factor. Asterisks (* p<0.05, ** p<0.01, *** p<0.001) indicate statistically significant differences between groups.



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Multiple signaling pathways convey central and peripheral signals to regulate pituitary function: Lessons from human and non-human primate models

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ABSTRACT

The anterior pituitary gland is a key organ involved in the control of multiple physiological functions including growth, reproduction, metabolism and stress. These functions are controlled by five distinct hormone-producing pituitary cell types that produce growth hormone (somatotropes), prolactin (lactotropes), adrenocorticotropin (corticotropes), thyrotropin (thyrotropes) and follicle stimulating hormone/luteinizing hormone (gonadotropes). Classically, the synthesis and release of pituitary hormones was thought to be primarily regulated by central (neuroendocrine) signals. However, it is now becoming apparent that factors produced by pituitary hormone targets (endocrine and non-endocrine organs) can feedback directly to the pituitary to adjust pituitary hormone synthesis and release. Therefore, pituitary cells serve as sensors to integrate central and peripheral signals in order to fine-tune whole-body homeostasis, although it is clear that pituitary cell regulation is species-, age- and sex-dependent. The purpose of this review is to provide a comprehensive, general overview of our current knowledge of both central and peripheral regulators of pituitary cell function and associated intracellular mechanisms, focusing on human and non-human primates.

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Contents

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1. Introduction: the complexity and versatility of actions of the pituitary gland

The pituitary gland, also known as the “master gland”, is a fundamental regulator of a plethora of relevant physiological functions such as growth, puberty, reproduction, lactation, metabolism and stress. To exert its function, the pituitary receives and processes the information originating from central and peripheral signals (as illustrated in Fig. 1) and appropriately conveys it to several, key target endocrine and non-endocrine organs (Musumeci et al., 2015). Thus, to achieve their goal, these complex networks of multiple regulatory signals must be integrated together to finely modulate the synthesis and release of various pituitary hormones, which, in turn, will be responsible to control the function of various organs involved in the vital processes mentioned above (Musumeci et al., 2015).

The pituitary gland is located at the *sella turcica*, a depression in the sphenoid bone, at the base of the brain (Lechan et al., 2000) and is comprised of the adenohypophysis [consisting of the anterior (subject of this review) and intermediate lobes] and the neurohypophysis (or posterior lobe), which are two distinct structures from the morphological and functional point of view, which display a strong developmental and functional interplay (Asa De Grootet al, 2000). The adenohypophysis develops from an upward invagination of the oral ectoderm, named the Rathke's pouch (Kelberman et al., 2009), which contains undifferentiated proliferative progenitors that differentiate into five hormone-producing cell types: growth hormone (GH)-producing or somatotrope cells, prolactin-producing or lactotrope cells, adrenocorticotropin (ACTH)-producing or corticotrope cells, thyrotropin (TSH)-producing or thyrotrope cells, and follicle stimulating hormone (FSH)/luteinizing hormone (LH)-producing or gonadotrope cells (Musumeci et al., 2015). Remarkably, the synthesis and release of these pituitary hormones (GH, PRL, ACTH, TSH, FSH and LH) and the subsequent fundamental actions on the numerous physiological processes cited above are finely tuned by an intricate interplay among many primary regulators (Fig. 1). Specifically, the actions of multiple central (mainly hypothalamic) and peripheral signals, with their specific receptors located at the pituitary cells, are directly orchestrated and integrated at the intracellular signal transduction level to subsequently regulate pituitary hormone secretion.

Classically, the primary control of pituitary hormone secretion was thought to reside in the hypothalamus. The hypothalamic hormones involved in pituitary cell regulation have changed during vertebrate evolution. For example, for somatotropes, somatostatin (SST), GH-releasing factor (GHRH), and PACAP are considered the main regulators in teleosts, amphibians and reptiles. In contrast, PACAP does not have an obvious role in birds and mammals, wherein GHRH and SST regulate GH secretion through a tight interplay (for review, see (Gahete et al., 2008a)). There are many other examples of evolutionary differences in the number and nature of regulatory molecules implicated in the control of species-dependent pituitary hormone synthesis and release. Although a plethora of data has been generated using non-primate models (rats, mice, etc.), more limited information has been generated in humans due to the obvious intrinsic research limitations to explore pituitary physiology; however, non-human primates have emerged as suitable tools to model human pituitary function. Therefore, the present review provides a comprehensive overview of the different central and peripheral regulators of pituitary function and their associated intracellular mechanisms, primarily focusing on studies performed in humans and non-human primates.

2. Non-human primates as suitable model for the study of human physiology

The vast majority of the knowledge gathered to date about the regulation of human pituitary cell function has been generated through the use of laboratory rodents and human (patho)physiological samples (such as fetal and tumoral cell cultures). However, despite the significant information generated using these approaches, there are still a number of aspects of the regulation of

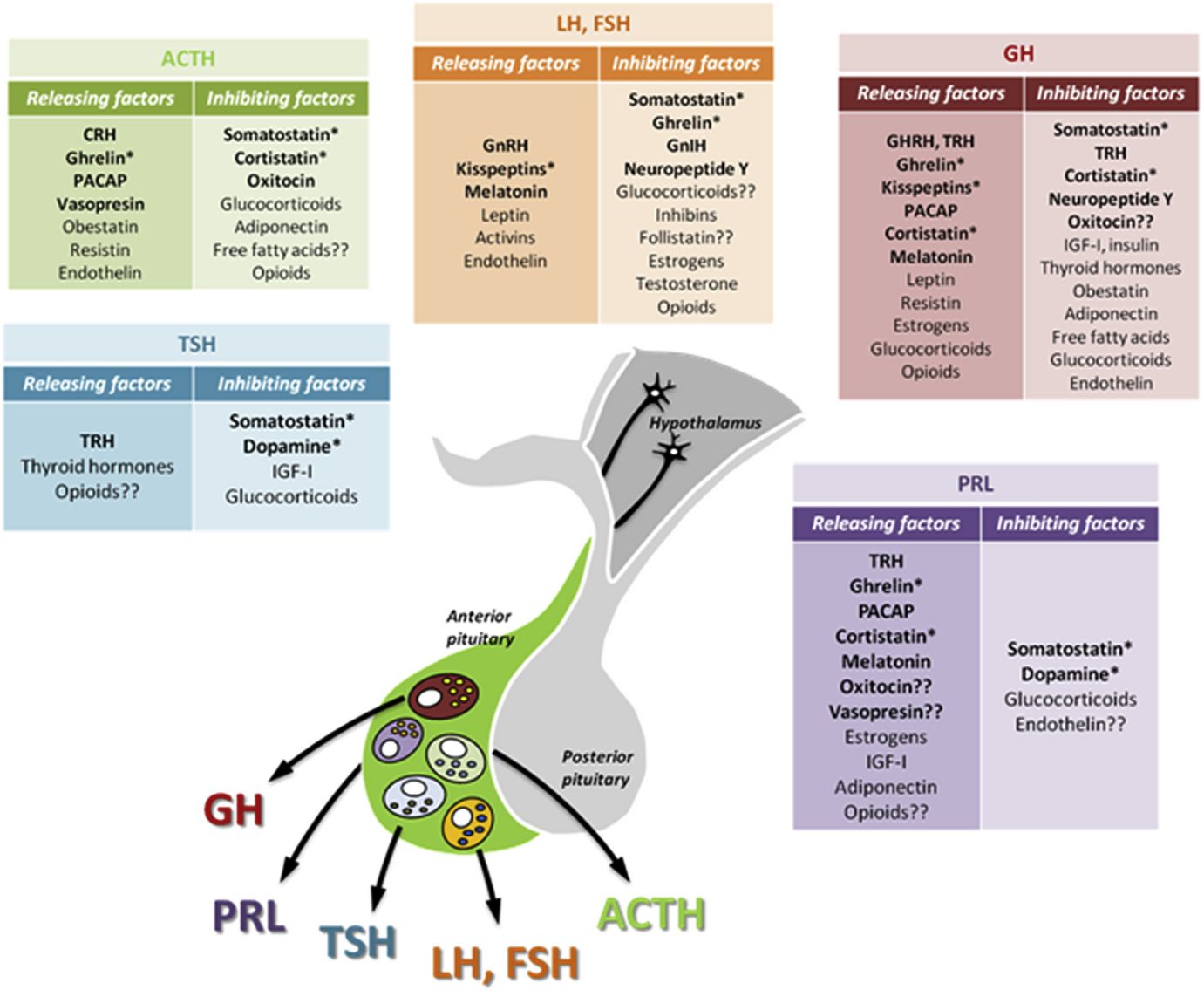

Fig. 1. Representative model summarizing central and peripheral regulators involved in the modulation of the function of different cell types comprising the anterior pituitary gland.

This model is based on the studies performed in human and non-human primates. Question marks (?) indicate regulators whose action have not been fully defined. Those factors shown in bold are primarily considered neuroendocrine factors, while those shown in standard type are considered coming from systemic sources. Those factors demarcated by asterisks (*) can be produced by central and systemic tissues.

normal adult human pituitary physiology that remain unclear. Therefore, our laboratory and others have used pituitary cells obtained from non-human primates. Specifically, baboons (*Papio sp*) and rhesus monkeys (*Macaca mulatta* and *Macaca fascicularis*), three species belonging to the *Cercopithecoidea* family (the Old World monkeys) (Perelman et al., 2011), are the most commonly used non-human primates in biomedical research. Indeed, results obtained from non-human primate models are used for translational research to humans (Braundmeier and Fazleabas, 2009; Comuzzie et al., 2003; McClure, 1984). Comparative genomic analyses exploring their molecular phylogeny and their evolutionary process have revealed that the separation of this family from the *Hominoidea* family occurred approximately 25 million years ago, which is relatively recent compared to the separation of rodent lineages with eutherian mammals that happened between 65 and 85 million years ago (Eizirik et al., 2001; Page and Goodman, 2001). Indeed, *Macaca mulatta* and *Macaca fascicularis* show a genetic identity of 93,54% and 92,83% with *Homo sapiens*, respectively (Gibbs et al., 2007; Ebeling et al., 2011). Additionally, the fact that olive baboon (*Papio anubis*) also shares a high fidelity at genomic, proteomic and physiological levels, together with the *in vivo* and *in vitro* conservation of the pituitary regulatory systems (as discussed below), makes these species suitable models to study the effects of different peptides/hormones on pituitary cell function, which cannot be evaluated in healthy human subjects. Hence, current evidence supports the notion that non-human primates can be considered as valuable and useful models to study normal, non-pathological, human physiology (Braundmeier and Fazleabas, 2009; Guardado-Mendoza et al., 2009; Kineman and Luque, 2007; Luque et al., 2006, 2014; Ibáñez-Costa et al., 2015).

3. Central modulators of pituitary cell function

3.1. Hypothalamic modulators of pituitary cell function

In 1965, it was shown for the first time that hypothalamic extracts induced GH release (Müller and Pecile, 1965). Since that time, the understanding of the neuroendocrine control of the somatotrope, as well as other pituitary cell types has led to the identification of a plethora of central factors that regulate pituitary function. Below is an overview of these central factors, shown in bold in Fig. 1.

3.1.1. GH-releasing hormone (GHRH)

GHRH is a 44-amino acid peptide hormone originally isolated and identified from a pancreatic tumour causing acromegaly (Guillemin et al., 1982; Rivier et al., 1982) and subsequently shown to be produced by neurons located in the arcuate nucleus (ARC) of the human hypothalamus (Ling et al., 1984). GHRH has been unequivocally accepted as the main hypophysiotropic neuropeptide in the generation and maintenance of pulsatile/episodic GH secretion in humans (Gahete et al., 2009; Murray et al., 2015), as well as in female rhesus monkey (Nakamura et al., 2003). Specifically, either GHRH antagonism or ARC nucleus ablation results in an impairment of GH pulsatility or complete loss of GH secretion, respectively (Murray et al., 2015; Goldenberg and Barkan, 2007). In addition, administration of synthetic GHRH reliably increases GH release in humans (Goldenberg and Barkan, 2007), as well as in female baboon pituitary cultures (*Papio anubis*) (Córdoba-Chacón et al., 2012). Previous studies have shown that GHRH specifically couples to GHRH receptor in somatotrope cells to activate multiple intracellular signaling mechanisms. Thus, in several species, including humans and baboons, it has been described that GHRH/GHRH-R coupling significantly stimulates GH release by activating adenylate cyclase (AC), increasing cAMP production, which in turn leads to an increase in protein kinase A (PKA) activity (Kineman and Luque, 2007; Córdoba-Chacón et al., 2012; Mayo et al., 2000). Additionally, it has been described that GHRH also requires other signaling pathways such as intracellular and extracellular Ca^{2+}, NOS/NO/GC/cGMP and/or PKC/PLC pathways to stimulate GH release in other species including non-human primates (Kineman and Luque, 2007; Gracia-Navarro et al., 2002).

3.1.2. Somatostatin (SST)

SST or somatotropin-release inhibitory factor (SRIF) is derived from a 116 precursor that produces two different cyclic forms by alternative post-translational processing: somatostatin-14 and somatostatin-28. SST biological actions are mediated by its specific interaction with at least 5 receptor subtypes ($SST_{1\text{-}5}$ receptors), which exhibit the structure of typical G-protein-coupled receptors (GPCRs) with seven transmembrane domains. Similar expression profile for all five receptor subtypes has been reported in human and baboon pituitary extracts and pituitary cell cultures, where subtypes 2 and 5 are the predominant subtypes (Neto et al., 2009; Córdoba-Chacón et al., 2011; Ibáñez-Costa et al., 2017a). Specific SST binding elicits the recruitment of several downstream transduction pathways including AC, protein phosphatases, cGMP dependent protein kinases, and calcium and other ion channels (Lim et al., 2000; Chang et al., 2012; Eigler and Ben-Shlomo, 2014). Overall, SST exhibits inhibitory actions on virtually all (neuro) endocrine secretions. At the anterior pituitary, SST is the main inhibitory signal for somatotrope function by directly inhibiting GH release as well as antagonizing the GH stimulatory effect elicited by either GHRH or ghrelin (Gahete et al., 2009; Lim et al., 2000). Interestingly, in non-human primates, it has been shown that SST can exert both negative and positive effects on GH release (Córdoba-Chacón et al., 2011). Specifically, it has been documented that high doses of SST do not alter basal GH secretion but block both GHRH- and ghrelin-induced GH release (Córdoba-Chacón et al., 2011). In contrast, low doses of SST significantly stimulate GH release, to a similar extent to that elicited by GHRH or ghrelin, in primary pituitary cultures from female adult baboons (*Papio anubis*) (Córdoba-Chacón et al., 2011). In this experimental model, SST inhibitory actions were shown to be mediated through activation of SST_1 and SST_2 receptors, which involved AC and MAPK signaling. In contrast, SST_5 receptor signaling through AC/cAMP/PKA and intracellular calcium pathways mediated the stimulatory action of low doses of SST on GH release. In addition, it has been reported that the main regulators of the hypothalamic-GH axis (GHRH, ghrelin and SST) in baboons can also regulate the expression of their receptors by both homologous and heterologous mechanisms (Córdoba-Chacón et al., 2012).

Besides the well described SST role on somatotrope function, it has also been documented that SST regulates other anterior pituitary cell types in several animal models, as well as humans (Theodoropoulou and Stalla, 2013). Specifically, in human fetal pituitary cultures, SST has been reported to exert an inhibitory effect on TSH, PRL and ACTH release, which mainly involves the differential participation of SST_2 and SST_5 receptors (Theodoropoulou and Stalla, 2013; Shimon et al., 1997). SST-mediated inhibition of PRL, ACTH, TSH, LH or FSH has also been described in healthy humans (Weeke et al., 1975; Faure et al., 1977; Hadjidakis et al., 1986; Yang et al., 1996; Bratusch-Marrain and Waldhäusl, 1979; Uberti et al., 1985). However, contradictory results have been published showing no significant effects of SST on spontaneous PRL or ACTH secretion (Gottero et al., 2004).

3.1.3. Ghrelin

Ghrelin is a 28-amino acid peptide hormone originally isolated from the stomach of humans and rats (Kojima et al., 1999) based on its strong GH-releasing ability, which is mediated through the activation of the GH-secretagogue receptor 1a (GHSR1a), first identified as an orphan GPCR and later identified as the receptor for synthetic GH-secretagogues. Soon after its discovery and isolation, ghrelin was also found to be present in multiple organs and tissues. At the level of the central nervous system, ghrelin expression has been detected in the pituitary and hypothalamus (Ueberberg et al., 2009; Ibáñez-Costa, 2015). Ghrelin circulates in two main forms, octanoylated (acylated) and deoctanoylated (deacylated). Acyl-ghrelin was the first form to be identified, based on its ability to stimulate GH release upon GHSR1a activation. In contrast, unacylated-ghrelin lacks the GH stimulatory action elicited by acyl-ghrelin on somatotrope cells. Although acylated ghrelin stimulates GH secretion directly acting on human (Tong et al., 2012) and monkey pituitaries (Kineman and Luque, 2007), an indirect hypothalamic-mediated mechanism involving an increase in GHRH and a weak inhibition of SST neurons, has also been documented (Kineman and Luque, 2007; Baragli et al., 2011; Lorenzi et al., 2009; Motta et al., 2016). In terms of signal transduction, ghrelin/GHSR1a interaction at the pituitary level has been shown to activate multiple signaling cascades, including phospholipase C (PLC), protein kinase C (PKC), PKA (Kojima and Kangawa, 2005), intracellular and extracellular Ca^{2+} or mitogen-activated protein kinases (Lorenzi et al., 2009; Mousseaux et al., 2006; Camiña et al., 2007). Interestingly, besides its action on somatotrope function, studies in humans and non-human primate models have revealed that ghrelin also regulates anterior pituitary function by inhibiting LH and FSH secretion and consequently modulating reproductive function (Motta et al., 2016; Lanfranco et al., 2008; Kluge et al., 2009), as well as stimulating PRL and ACTH release (Motta et al., 2016; Takaya et al., 2000; van der Lely et al., 2004; Coiro et al., 2005; Lengyel, 2006; Messini et al., 2011).

3.1.4. Pituitary adenylate cyclase activating polypeptide (PACAP)

PACAP is a C-terminally amidated peptide with two forms of 38 and 27 residues, which belongs to the VIP/secretin/glucagon superfamily of peptides. It was first isolated from ovine hypothalamic extracts based on its ability to stimulate AC activity in rat pituitary cells. In mammals, contradictory findings about the role of PACAP on GH secretion have been documented. Some studies report a stimulatory action while others, show no effect on GH release (Gahete et al., 2009). In human somatotrope tumour cells, PACAP was able to increase both cAMP production and GH release similarly to that previously reported for GHRH although in a less potent manner (Adams et al., 1994). This stimulatory action was shown to involve the activation of voltage-operated/gated Ca^{2+}channels via AC/PKA pathway (Gahete et al., 2009; Murakami et al., 2001). Conversely, in healthy human volunteers, intravenous PACAP administration was unable to induce GH and gonadotropins release (Chiodera et al., 1996). However, it has been described that intravenous PACAP administration can regulate ACTH and PRL secretion in different mammalian species including humans (Chiodera et al., 1996; Thomas et al., 2012; Halvorson, 2014). PACAP elicits its biological action by coupling to different G-protein-coupled receptors classified into three groups based on their differential affinity for PACAP or VIP. Thus, PACAP type 1 receptors (PAC1R) are more specific for PACAP while VPAC1 and VPAC2 receptors present similar affinity for either PACAP isoforms or VIP. Additionally, PAC1R alternative splicing generates at least five different PAC1R subtypes that seem to trigger different signaling pathways as well as their relative affinity for PACAP isoforms (Halvorson, 2014; Kanasaki et al., 2016). All these receptors are widely distributed throughout the brain, including hypothalamus and pituitary, as well as in peripheral organs (Halvorson, 2014; Kanasaki et al., 2016).

3.1.5. Gonadotropin releasing hormone (GnRH)

GnRH is a hypothalamic decapeptide released in a pulsatile manner that is essential in the maintenance of reproductive function throughout the episodic secretion of gonadotropic pituitary hormones (Krsmanovic et al., 2009). Indeed, the direct effect of GnRH on LH and FSH release in healthy subjects is firmly established (Jayasena et al., 2015). In line with this, a stimulation of LH secretion in response to exogenous GnRH has been also reported in macaques (Weinbauer et al., 1992). Additionally, a significant increase on LH secretion after GnRH treatment has been observed in primary pituitary cell cultures from baboons. In most vertebrates, including humans, at least two GnRH receptor (GnRH-R) isoforms have been described, type I and type II GnRH-R. Both isoforms are expressed at the pituitary and non-pituitary level, including reproductive and non-reproductive tissues. Type I GnRH-R is the functional receptor isoform that belongs to the G-protein-coupled receptor superfamily with seven transmembrane domains, a hydrophilic extracellular domain and a hydrophobic cytosolic tail. This GnRH-R differs from others GPCRs in its short cytosolic tail that slows receptor internalization and prevents rapid desensitation (Choi et al., 2012). Human type II GnRH-R is a non-functional isoform due to the presence of a frameshift and a premature stop codon in its sequence (Choi et al., 2012; Limonta et al., 2012). The signaling pathways involved in the GnRH actions cited above were NOS/NO/GC/cGMP pathway and extracellular Ca^{2+} mobilization but not AC pathway (Luque et al., 2011). On the other hand, in higher vertebrates, including humans and non-human primates, no data have been documented on the effect of GnRH on GH release under normal conditions, while several studies documented a modulatory effect of GnRH on GH release under different pathological disorders (Skinner et al., 2009).

3.1.6. Kisspeptins

Kisspeptin (KISS1) is an amidated neurohormone first identified as a key regulator involved in GnRH control at the level of the hypothalamus. KISS1 gene encodes a 145-amino acids precursor protein that can originate four possible derivate peptides with 54-, 14-, 13- or 10-amino acids (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Stafford et al., 2002). All these peptides have the same efficacy and affinity for their receptor, GPR54, being kisspeptin-10 the most commonly used in biomedical research (Ramaswamy et al., 2009; Smith et al., 2008). In addition to its central effects, KISS1 and its receptor (GPR54, KISS1R or AXOR12) are widely distributed in different tissues including pituitary gland, suggesting that this neurohormone system could play an important role in the control of hypophyseal hormone release (Kotani et al., 2001; Muir et al., 2001; Ramaswamy et al., 2009; Clarkson et al., 2009; Pinilla et al., 2012; Roa et al., 2011). Specifically, in non-human primates (*Macaca mulatta*), kisspeptin-positive cells have been described to be present in intermediate lobe co-localizing with α-MSH, in neural lobe with GnRH axons, and, only in 50% with ACTH-positive cells in the periphery of anterior lobe of pituitary (Ramaswamy et al., 2009; Gahete et al., 2016). In humans, kisspeptin-54 and kisspeptin-10 were able to similarly induce LH and FSH levels. However, both kisspeptins were less potent in stimulating gonadotropins levels than GnRH (Jayasena et al., 2015; Narayanaswamy et al., 2016). Furthermore, intravenous administration of kisspeptin-10 in *Macaca mulatta* increased LH levels, an effect apparently mediated by hypothalamic actions (GnRH-induced), while other hypophyseal hormones were not altered (Ramaswamy et al., 2009). In addition, results from studies on women did not confirm a role of kisspeptin on GH, TSH and PRL release after acute or chronic administration (Jayasena et al., 2014). However, kisspeptins seem to exert a direct effect on primary pituitary cell cultures from baboons. Specifically, kisspeptin-10 stimulated GH and LH secretion and mRNA levels after short- and long-term exposure (4–48 h), at a broad range of doses (10^{-14} to 10^{-6}) (Luque et al., 2011). In contrast, kisspeptin-10 did not alter FSH, PRL, ACTH or TSH secretion/expression. The signaling pathways involved in the regulation of GH and LH pituitary hormones by kisspeptins were phospholipase C, protein kinase C, MAPK, and intracellular Ca^{2+} mobilization. Interestingly, LH, but not GH, release also involved mammalian target of rapamycin (mTOR) and PI3K (Luque et al., 2011). Taken together, *in vitro* and *in vivo* evidences suggest that kisspeptins could play a relevant role, at least, on LH regulation and seems to exert the effects not only through hypothalamic actions but also directly on the pituitary gland.

3.1.7. Gonadotropin-inhibitory hormone (GnIH)

Gonadotropin-inhibitory hormone (GnIH) was initially discovered in the quail hypothalamus, wherein its inhibitory action on gonadotropin secretion from cultured anterior pituitary cells was documented (Tsutsui et al., 2000, 2017). Subsequent studies described that avian GnIH was well conserved across various mammals and primates including humans, in which an inhibitory action on reproductive function was also reported for these GnIH orthologs (Tsutsui et al., 2000, 2017). In particular, the functional human GnIH ortolog, RFRP-3, as well as other GnIH peptides are called RF-related peptides (RFRPs) in that they share a common structural feature with kisspeptins: the presence of a C-terminal Arg-Phe-NH2 (RFamide) motif, thus belongings to the RFamide peptide family members. In humans and non-human primates, GnIH/RFRP neural cell bodies are located at the dorsomedial region and intermediate periventricular nucleus of the hypothalamus, respectively. In addition, human GnIH/RFRP expression in cell bodies was also documented in other areas of the brain and in neuronal fibres projected to the median eminence (Ubuka et al.,

2009a, 2009b; Bentley et al., 2010; Ubuka et al., 2012; Clarke and Parkington, 2014). GPR147 (NPFF1, OT7T022) has been identified as the cognate receptor that mediates GnIH/RFRPs inhibitory actions. In this sense, it has been reported in a rodent ovarian cell line that RFRPs action reduces intracellular cAMP levels, suggesting that GPR147 couples to Gi protein (Hinuma et al., 2000). Additionally, in a mouse gonadotrope cell line, it has been reported that the inhibitory action of RFRPs on gonadotropin secretion is mediated by the inhibition of AC/cAMP/PKA/ERK pathway (Son et al., 2012). Moreover, human RFRP-3 is able to inhibit, *in vivo* and *in vitro*, GnRH-induced gonadotropin release in sheep through inhibition of intracellular calcium mobilization (Clarke and Parkington, 2014).

3.1.8. Corticotropin-releasing hormone (CRH)

CRH is a 41-amino acid peptide hormone produced by neuroendocrine cells of the paraventricular nucleus of the hypothalamus. At the anterior pituitary, CRH induces ACTH secretion, which, in turn, stimulates the secretion of glucocorticoid hormones (mainly cortisol in humans) from the adrenal cortex. CRH exerts its biological actions by coupling to specific receptors that recruit several intracellular effectors such as cAMP and protein kinases (Grammatopoulos, 2012). In addition, a role for CRH on somatotrope function/GH release has also been described in lower vertebrates (Rousseau et al., 1999; Rousseau and Dufour, 2007). However, in humans and non-human primates, in the absence of pathological conditions, there is not much evidence of such effect to date. Interestingly, in patients suffering from acromegaly, two independent groups have previously reported an increase in circulating GH concentration after treatment with either CRH or dexamethasone (DEX, a synthetic glucocorticoid). However, such stimulatory effect on GH has not been confirmed by other studies (Gahete et al., 2009). On the other hand, it has been suggested a role for CRH in the regulation of gonadotropin secretion based on the presence of its receptor in pituitary gonadotropes (Kageyama, 2013). However, these results are not conclusive due to the fact that CRH infusion in male rhesus macaques produced a clear increase on ACTH and cortisol levels, but the LH levels were not different from those observed in untreated control macaques (Norman, 1993).

3.1.9. Thyrotropin-releasing hormone (TRH)

TRH is a short neuropeptide (pGlu-His-Pro-NH2) initially isolated from hypothalamic extracts based on its ability to stimulate the release of thyroid-stimulating hormone. In mammals, it has been documented that TRH not only stimulates TSH but also PRL and GH release, although with species-specific differences (Gahete et al., 2009; Kanasaki et al., 2015). In humans, TRH induction induces GH release in adenomatous cell cultures from acromegalic subjects (Gahete et al., 2009; Okinaga et al., 2005). Under this experimental setting, TRH-induced GH release was dependent on the calcium influx through L-type calcium channels, with an attenuation in such calcium events elicited by a PKC inhibitor (Okinaga et al., 2005). In lactotrope cells, activation of the TRH receptor by TRH recruits the participation of Gq protein and stimulation of IP production, which in turn activates PKC pathway as well as the release of Ca^{2+} from different stores. Other signaling mechanisms triggered by TRH action includes ERK and MAPK (Kanasaki et al., 2015).

3.1.10. Neuropeptide Y (NPY)

NPY is a 36-amino acid peptide widely distributed throughout the central nervous system, with highest density of producing-neurons located at the hypothalamic arcuate nucleus (Adams et al., 1987). In some mammalian species, NPY seems to elicit a stimulatory effect on GH secretion (Gahete et al., 2009). NPY actions are mediated by multiple receptors that belong to the GPCR family (Pedrazzini et al., 2003). NPY administration to patients with prolactin-secreting pituitary adenomas, significantly increased GH levels in 60% of patients. However, in a different study, NPY administration did not alter GH release when administered to healthy young men (Lim et al., 2000). In several animal species, it has been described that NPY indirectly regulates different pituitary hormones secretion by acting first at the hypothalamic level by regulating the activity of GnRH, CRH, TRH and GHRH neurons (Pedrazzini et al., 2003; Gaikwad et al., 2005). In fact, it was described that administration of human NPY to the third cerebroventricle in ovariectomized (OVX) rhesus monkeys produced a marked LH suppression through the alteration of GnRH/LH secretory system (Kaynard et al., 1990). Moreover, it was also shown that NPY acts at the level of the median eminence to stimulate the release of GnRH or directly enhancing the LH secretion in response to GnRH through the transportation into the hypophyseal portal blood. Both of these mechanisms seems to involve the mobilization of intracellular calcium (Kalra and Crowley, 1992).

3.1.11. Dopamine (DA)

It has been previously reported that either DA precursor or DA agonist administration stimulated GH release in humans when administered subcutaneously, while decreased blood PRL concentration (Lal et al., 1973). However, such effect was partially or totally antagonized by an alpha-adrenergic component in monkeys and humans (Müller et al., 1999). Conversely, inhibitory actions of DA on GH release have also been reported (Müller et al., 1999; Garcia-Tornadú et al., 2010). To date, five DA receptors ($D_{1\text{-}5}$ receptors) coupled to diverse downstream signaling pathways have been described (Beaulieu and Gainetdinov, 2011). Lactotropes present the highest expression level of DRD2 while, in somatotropes, the DRD2 expression is significantly lower to that observed in adenomatous somatotropes (Neto et al., 2009; Taboada et al., 2011; Ben-Shlomo et al., 2016). Hetero- or oligomerization of SST receptors and DRs has been studied in non-pituitary cell models and was thus suggested as a molecular mechanism in somatotrope cells for the inhibition of GH release (Ben-Shlomo et al., 2016). In addition, DRD2 expression was also found in a high percentage of other pituitary cells, thus indicating that DRD2 expression is not confined to lactotrope cells. Consistent with the broad pituitary expression of DRs, one study reported DA can regulate ACTH release (Pivonello et al., 2007). Although DA receptors have been widely associated with multiple signaling pathways (Beaulieu and Gainetdinov, 2011), to the best of our knowledge the specific routes responsible of DA effects on human or primate pituitary gland remain to be determined.

3.1.12. Oxytocin (OT) and arginine-vasopressin (AVP)

OT and AVP are two hypothalamic hormones well known to exert post-hypophyseal (systemic) actions. However, OT and AVP have been also related with the modulation of anterior pituitary hormones, which could be anticipated by the high concentrations of both neurohormones found in the hypophyseal portal blood of non-human primates (Plotsky, 1987; Zimmerman et al., 1973). Indeed, AVP administration increases ACTH levels in healthy humans, wherein AVP seems to enhance CRH-stimulated ACTH release (Liu et al., 1983; DeBold et al., 1984; Hensen et al., 1988; Meller et al., 1991; Erfurth et al., 1996). In fact, it was reported that AVP from pituitary portal circulation is more important altering ACTH levels than AVP derived from peripheral circulation. Similarly, a stimulation of GH secretion has also been related with AVP infusion in human and non-human primates (Meller et al., 1991; Meyer and Knobil, 1966; Gagliardino et al., 1967; Brostoff et al., 1968; Chiodera and Coiro, 1985) and probably these effects

are mediated through stimulation of cholinergic-muscarinic mechanisms and/or mediated in part through catecholamines (Heidingsfelder and Blackard, 1968; Coiro et al., 1985). Regarding PRL secretion, it was reported a significant increase on PRL release after AVP administration compared with saline infusions (Erfurth et al., 1996). However, these results are not in agreement with other reports where no alterations of PRL levels were observed in response to AVP (Meller et al., 1991; del Pozo et al., 1980; Chiodera et al., 1988). Finally, the rest of anterior pituitary hormones do not seem to be significantly affected by AVP infusion in humans (Erfurth et al., 1996; Chiodera et al., 1988).

On the other hand, OT has been described to exert the opposite role of AVP on ACTH secretion in humans. In this regard, several studies have reported an inhibition of basal and stimulated ACTH release in normal human subjects (Legros et al., 1982; Legros et al., 1984; Gibbs, 1986; Chiodera and Coiro, 1987; Page et al., 1990). In contrast, other reports have not found changes on plasma ACTH levels after increasing doses of OT in men even when the OT doses and administration routes were the same as the studies mentioned above (Lewis and Sherman., 1985; Nussey et al., 1988; Coiro et al., 2011). In line with this, OT infusions did not alter basal or CRH-induced ACTH release in women, but was able to inhibit the potentiating effect of AVP on CRH-stimulated ACTH release (Suh et al., 1986). Regarding other anterior pituitary hormones, OT administration did not relevantly alter GH, PRL, TSH or LH and FSH responses in healthy humans (del Pozo et al., 1980; Page et al., 1990; Coiro et al, 1987, 2011; Chiodera et al., 1984a). However, other studies reported no changes on basal GH release, but a significant reduction on AVP-stimulated GH secretion (Chiodera et al., 1984b). Additionally, OT administration enhanced PRL release in response to vasoactive intestinal polypeptide (Chiodera et al., 1998) and TRH in women (Coiro et al., 1987).

Although the above data demonstrate AVP and OT can mediate anterior pituitary hormone secretion, *in vitro* data is lacking whether these effects are direct or also represent the combined actions of these peptides on central neuronal function, which may in part help to explain the contradictory results currently available.

3.2. Other central modulators of pituitary cells function

3.2.1. Melatonin

Melatonin (MT) or N-acetyl-5-methoxy tryptamine is a hormone produced by the pineal gland. The presence of MT receptors at the pituitary gland suggested a possible influence of this hormone on the regulation of anterior pituitary hormones (Weaver et al., 1993; Wu et al., 2006). Indeed, the secretion of pituitary hormones show a circadian rhythm (Landgraf et al., 1982) and it has been suggested that these patterns could be a consequence of nocturnal MT secretion (Forsling et al., 1999). Specifically, *in vivo* studies suggest the influence of melatonin on the secretion of GH and other pituitary hormones in primates and healthy humans (Brandon et al., 2002; Lisoni et al., 1986; Smythe and Lazarus, 1973; Wright et al., 1986; Kostoglou-Athanassiou et al., 1998). However, the available data is not consistent. First, MT had a different effect depending on the stage of human growth. In infants, diurnal cycles seem to be beneficial for growth, which suggests a negative correlation between MT and GH at this age (Brandon et al., 2002). At puberty, oral administration of MT treatment resulted in decrease GH (Lisoni et al., 1986), which may explain a greater growth in this age range in summer when MT levels are lower (Smythe and Lazarus, 1973). On the other hand, in adults, MT administration increased basal GH levels (Wright et al., 1986) and seemed to increase sensitivity to GHRH via altering the SST inhibitory pathway. However, other studies have shown that in young men, MT does not influence GH release but correlates with PRL and cortisol (Kostoglou-Athanassiou et al., 1998), which was also observed by others in both women and men (Bispink et al., 1990; Rao and Mager, 1987). Exogenous MT administration can also influence PRL, LH and TSH secretion in women (Terzolo et al., 1993), wherein MT could lead to hyperprolactinemia (Okatani and Sagara, 1993). In men, MT administration has also been associated with a reproductive role, by regulating LH and FSH secretion. Particularly, MT increases LH amplitude pulse in a dose-independent manner without altering FSH values (Cagnacci et al., 1991) and its decrease may lead to sterility (Li and Zhou, 2015; Luboshitzky et al., 2000). Interestingly, the effect of MT on pituitary secretions seems to be dose- and time-dependent, in that MT causes an increase on neurohypophysial hormones (AVP and OT) and GH at low doses (0,5 mg), whereas at high doses (5 mg) the only GH levels are impacted (Forsling et al., 1999). In addition, an acute MT administration increases GH levels (Smythe and Lazarus, 1974; Valcavi et al., 1987) and modulates the secretion of other pituitary hormones (LH and/or PRL) in men and women (Ninomiya et al., 2001; Perras et al., 2005; Waldhauser et al., 1987). Surprisingly, chronic MT administration, does not cause any effect on GH (Wright et al., 1986). Studies performed on non-human primates have shown that MT only was able to slightly affect the insulin-stimulated GH release without producing any change in basal or stimulated PRL, TSH, LH or FSH secretion (Chrousos et al., 1982). However, it has been recently described the role of MT on primary pituitary cell cultures obtained from baboons (*Papio anubis*), where MT showed clear stimulatory actions on GH and PRL expression and secretion in a dose and time-dependent manner through common and distinct signaling pathways. Specifically, the effects of MT on GH and PRL levels were mediated through AC/PKA and extra-/intra-cellular calcium pathways, although the effects on GH, but not PRL release also required the activation of PLC route. Regarding other pituitary hormones, MT did not produce any change on ACTH, LH, FSH or TSH synthesis or release on baboon primary pituitary cell cultures (Ibáñez-Costa et al., 2015). Finally, it has been suggested that the action of MT at the pituitary level could be mediated through the MT1 receptor (Ibáñez-Costa et al., 2015; Dubocovich and Markowska, 2005).

3.2.2. Cortistatin (CORT)

CORT is a neuropeptide produced by post-translational cleavage, which can lead to the generation of two mature products CORT-17 and CORT-29 in humans (Broglio et al., 2002a). CORT, as well as SST, is distributed and expressed in wide variety of human and rodent tissues (including pituitary gland), even more than that previously assumed (Dalm et al., 2004; Cordoba-Chacón et al., 2016). Additionally, CORT shares with SST a high structural homology that explains their similar capacity to bind the same family of receptors (SST_{1-5} receptors) (de Lecea et al., 1996; Fukusumi et al., 1997; Siehler et al., 1998, 2008; Spier and de Lecea, 2000). Despite the structural and functional similarities of these molecules, they display crucial differences (Broglio et al., 2008; de Lecea and Castano, 2006), including the capacity of CORT, but not SST, to bind to other receptors such as GHSR1a (Deghenghi et al., 2001, 2003), or Mrgx2 (an orphan G-protein-coupled receptor belonging to Mas-related genes family) (Robas et al., 2003). Also CORT is able to mediate different/opposite actions compared to SST such as the effect on immune cells, the increase on slow wave sleep, the reduction in the synthesis of inflammatory mediators, as well as differential effects on pituitary function (see below) (Gahete et al., 2008b). At the pituitary level, CORT inhibits GH release through the activation of SST receptors in young males and, indeed, CORT and SST show equal inhibition of GH release induced by GHRH, ghrelin and synthetic analogues (Gottero et al., 2004; Broglio et al., 2002a, 2002b; Benso et al., 2003; Ibáñez-Costa et al., 2017b). However, CORT, as well as SST, did not affect ghrelin-stimulated

PRL, ACTH and cortisol levels (Broglio et al., 2002a) even when both showed the same inhibitory effect on ghrelin release (Broglio et al., 2002b). Interestingly, CORT-8, a synthetic CORT-analogue that binds GHSR1a but not SST receptors, was not able to modulate ghrelin- or hexarelin-stimulated GH, PRL and ACTH release, suggesting a predominant role of SST receptors on the known actions of CORT on GH release (Prodam et al., 2008), which seems to be further supported by *in vitro* studies (Deghenghi et al., 2001; Muccioli et al., 2001). Indeed, *in vitro* observations in human fetal pituitary cells using CORT showed an inhibitory effect on GH release, which was even greater than that elicited by SST (Ibáñez-Costa et al., 2017b; Rubinfeld et al., 2006). In female baboons (*Papio anubis*), CORT blunted GH and ACTH basal secretion and also decreased GH and POMC mRNA expression. Surprisingly, CORT stimulated, while SST inhibited, PRL release in baboon primary pituitary cell cultures without altering mRNA expression. This stimulatory effect seems to be mediated through GHSR1a, since the treatment with an antagonist of this receptor completely blocked this stimulatory response to CORT (Córdoba-Chacón et al., 2011; Ibáñez-Costa et al., 2017b). Finally, in primate pituitary cell cultures, low concentrations of both CORT-17 and SST-14 (10^{-17} and 10^{-15} M) are able to stimulate GH release through SST_5 receptor requiring activation of AC/cAMP/PKA and intracellular Ca^{2+} pathways. Therefore, all this information indicates that CORT directly modulates the function of different pituitary cell types and these actions in humans and non-human primate models are dose- and cell type-dependent and receptor-specific (Córdoba-Chacón et al., 2012).

4. Peripheral modulators of pituitary cell function

4.1. Glucocorticoids (GCs)

Glucocorticoids, the end products of the CRH (hypothalamic) −ACTH (pituitary)- adrenal axis, negatively feedback to suppress its own axis function, where many reports demonstrate GC suppress ACTH secretion *in vivo* and in primary pituitary cell cultures (Waltman et al., 1991; Arvat et al., 1998; La Marca et al, 1999; Roelfsema et al., 2016). GCs have also been shown to regulate GH secretion (Mazziotti and Giustina, 2013) *in vitro* and *in vivo* in humans and non-human primates (Luque et al., 2006; Kohler et al., 1968; Mulchahey et al., 1988). *In vivo* observations in healthy humans support the hypothesis that GCs are able to stimulate or inhibit GH secretion depending on the specific conditions (dual effect) (Mazziotti and Giustina, 2013; Pinto et al., 1999; Giustina et al., 1990a; Casanueva et al., 1990; Burguera et al., 1990; Kaufmann et al., 1988). Especially, during short-term incubations (1 h), GCs produce an inhibition of GHRH-stimulated GH secretion probably due to an increase of endogenous SST secretion (Giustina et al., 1990a). This inhibitory effect was corroborated using acetylcholinesterase inhibitors, which are known to elicit GH secretion through a decrease in the hypothalamic release of SST (Massara et al., 1986; Ghigo et al., 1987; Locatelli et al., 1986). Thus, the presence of acetylcholinesterase inhibitors, alone or in combination with GHRH, blocked the inhibitory effect of GCs on GH release (Giustina et al., 1990b). In contrast, a rise of GH values was detected after 3 h treatment with DEX (iv. or oral administration) in normal subjects. Interestingly, after 12 h incubation with DEX, the GH release was again inhibited (Casanueva et al., 1990; Burguera et al., 1990). In fact, the GC prednisone was able to blunt GHRH-stimulated GH response after 4 days of treatment in healthy subjects (Kaufmann et al., 1988). Although the mechanisms behind these effects are not yet clear, there are potential reasons that could explain these responses: 1) a rise of GHRH secretion and inhibition of negative feedback of IGF-I in a short period of treatment; 2) a stable increase of SST release due to a sustained hypercortisolemia and; 3) the time of administration. In contrast, the effects of GCs on PRL secretion in healthy humans are still unclear inasmuch as several reports showed a suppression on basal and TRH-stimulated PRL levels after DEX administration (Dussault, 1974; Sowers et al., 1977), which is in accordance with *in vitro* results described in baboon (Steger et al., 1981). However, TRH-stimulated, but not basal, PRL is reduced by DEX in women (La Marca et al, 1999), and no effect on basal or stimulated PRL was found in normal subjects (Re et al., 1976). These differences between studies could be due to the dose of GCs, route of administration, experimental design or even sensitivity limit of PRL radioimmunoassays. In addition, GCs have been shown to alter TSH secretion in humans, where clear inhibition has been observed in baseline and TRH-stimulated levels in response to a short or a long-term GCs treatment and this suppression was reflected by a fall in T_3 concentration in adults and preterm infants (Sowers et al., 1977; Re et al., 1976; Wilber and Utiger, 1969; Otsuki et al., 1973; Faglia et al., 1973; Barbieri et al., 1985; Brabant et al., 1987; Shimokaze et al., 2012). The use of hypothalamic somatostatinergic and dopaminergic inhibitory compounds revealed that these mechanisms are involved in the TSH response to GCs treatment (Coiro et al., 2000). In addition to ACTH and GH, GCs also modulated LH and FSH levels in humans. It has been reported that DEX cannot alter basal LH or FSH, but decreased LH levels after GnRH stimulation, but not confirmed in another study (Sakakura et al., 1978; Veldhuis et al., 1992).

Interestingly, one of the first evidences showing the direct effect of GCs on GH secretion *in vitro* was the demonstration of a marked increase of GH production after the treatment with cortisol in primary pituitary cell cultures obtained from *Macaca mulatta* (Kohler et al., 1968). Interestingly, the use of an inactive analogue (11 α-hydroxycortisol) blunted the GH response, and the mechanisms behind this effect involved protein and probably RNA synthesis (Kohler et al., 1968). In line with this, treatment with DEX also produced a significant increase in GH secretion when fetal rhesus monkey pituitary cells were treated (Mulchahey et al., 1988). These results have been corroborated in another primate model, *Papio anubis*, in which DEX and hydrocortisone (HY) caused a clear increase of GH release in primary pituitary cell cultures after a 24 h incubation period. Moreover, both GCs significantly stimulated GH, GHRH-R and GHS-R mRNA levels in baboon primary pituitary cell cultures, which could suggest that the increase in GH mRNA is translated into an increase of GH production and secretion (Luque et al., 2006). Furthermore, similar results were obtained in cultures of normal human pituitaries from patients with metastatic breast carcinoma (Bridson and Kohler, 1970) and in human fetal anterior pituitary cell cultures (Mulchahey et al., 1988). In both cases, GCs (cortisol or DEX) were able to produce a marked increase of GH release under basal and GHRH stimulated conditions in a time-dependent manner (Mulchahey et al., 1988; Bridson and Kohler, 1970). In contrast, in the case of PRL secretion, different concentrations of cortisol significantly decreased PRL secretion in tissue fragments from baboon pituitary glands even when TRH was used to stimulate PRL release (Steger et al., 1981). To date, the vast knowledge about the mechanisms and signaling pathways underlying these effects have been described mainly in rodents and involve the activation of cAMP/PKA or PKC signaling pathways and intracellular free calcium mobilization (Shipston, 1995); however, whether the actions of GCs on human or primate pituitary hormone secretions are mediated through these signaling pathways remains to be fully elucidated.

4.2. Thyroid hormones (THs)

THs are produced and secreted by the thyroid gland and are

mainly regulated by thyrotropin (TSH). Likewise, THs regulate TSH through a direct negative feedback on pituitary gland (Brabant et al., 1987). In this sense, T3 and T4 administration significantly reduced serum TSH levels without any alteration on its pulsatility in healthy humans (Brabant et al., 1987). Moreover, TRH-stimulated TSH response can be suppressed by THs alone or by T3 combined with ipodate (iodinated radiocontrast agent that inhibits the conversion of T4 to T3). Conversely, combination of T4 and ipodate did not alter the TSH response to TRH. These results suggest that the conversion of T4 to T3 could be important for the THs feedback action (Brabant et al., 1987; Wenzel et al., 1975). In this regard, it is important to mention that among thyroid hormone analogues such as tetraiodothyroaceticacid (TETRAC) or triiodothyroaceticacid (TRIAC), only TRIAC is known to be able to partially inhibits the synthesis and secretion of TSH and PRL in normal subjects (Medeiros-Neto et al., 1980; Menegay et al., 1989). Interestingly, THs also seem to play a role in the regulation of GH as several studies have described that an increase in THs levels in humans is able to produce a strong reduction of pituitary GH release probably due to a rise of hypothalamic SST tone (which blunted any stimulatory effect) or, to a reduction on GHRH release (Giustina and Veldhuis, 1998; Giustina and Wehrenberq, 1995). However, THs could also play a direct role in the regulation of somatotropes as T3 can decrease the expression of hGH gene in transfected GC cell cultures (Cattini et al., 1986). Moreover, the negative effect of T3 on GH secretion was also described in pituitary cultures from fetal rhesus monkey and humans. Specifically, treatment of rhesus monkey cells with T3 produced a significant inhibition of GH release after GHRH stimulation but did not alter basal GH secretion (Mulchahey et al., 1988). Conversely, the results with human cells showed a strong reduction of basal and GHRH-stimulated GH secretion (Mulchahey et al., 1988). In the same line, T3 treatment was also able to decrease hGH RNA levels without a clear effect at the protein level in transgenic (171hGH/CS-TG) mice expressing the human GH gene (Vakili et al., 2011). However, to the best of our knowledge, the signaling pathways and mechanisms associated with the effects of THs and its analogues in humans and non-human primate pituitaries have not been identified.

4.3. Insulin and IGF-I

Insulin/IGF-I system comprises a complex family of related peptides, membrane receptors and high-affinity IGF binding proteins (IGFBP) (Jones and Clemmons, 1995; Rajaram et al., 1997), which have been directly associated with a strong regulation of pituitary cell function in several models (Gahete et al., 2013; Yamashita and Melmed, 1986). Indeed, IGF-I and IGFBP-3 are positively correlated with spontaneous 24 h GH secretion (expressed as AUC) in different healthy humans subgroups (sex or pubertal stage) (Blum et al., 1993). IGF-I in turn acts via negative feedback to the hypothalamus, as well as the pituitary to control GH secretion. For example, low doses of recombinant IGF-I infusion were able to blunt the fasting-stimulated GH secretion in men fasted for 32 h (Hartman et al., 1993). In the same line, the administration of recombinant IGF-I at physiological doses diminished GH response to GHRH without any alteration on spontaneous GH levels (Ghigo et al., 1999). Moreover, it has been shown that circulating free and not total IGF-I could be a key mediator of GH secretion since the rise of GH levels after 24 h was negatively correlated with the reduction of free IGF-I (Chen et al., 2005). However, a single dose of recombinant IGF-I is not sufficient to alter basal or pulsatile GH release or impact FSH, LH and PRL levels, but does suppress TSH (Trainer et al., 1994). This discrepancy between different studies could be due to the dose or route of administration.

Insulin infusion, like IGF-I, has been shown to reduce GH response to GHRH in healthy humans (Lanzi et al., 1997). Also, an increase of insulin concentration observed in healthy humans undergoing overeating, is accompanied by a reduction of GH levels (Cornford et al., 2011). The action of both IGF-I and insulin could be in part due to direct suppression of somatotrope function. Specifically, IGF-I and IGF-II dose dependently decreased GH release in both fetal and adults cultures (Goodyer et al., 1986). In that same study, IGF also reduced PRL levels in adult, but not in fetal pituitary cultures, while having no impact on ACTH or LH release (Goodyer et al., 1986). In another study, IGF-I was able to suppress GH mRNA levels induced by cAMP plus hydrocortisone and, to reduce stimulated GH secretion without altering basal GH secretion in human choriocarcinoma cells transfected with hGH gene (Yamashita et al., 1987). Moreover, a suppression of somatrotrope function has been reported in baboon primary pituitary cell cultures wherein IGF-I was able to significantly blunt GH release and mRNA levels in a dose-dependent manner after 24 h of treatment. Like IGF-I, insulin also inhibited GH secretion and mRNA levels at physiological concentrations in baboon primary pituitary cell cultures although with a different dose-dependent pattern (Luque et al., 2006). In another study by the same group, the inhibitory actions of insulin and IGF-I required distinct intracellular signaling pathways to suppress somatotrope function in baboon pituitary cell cultures (i.e. IGF-I acted through PI3K, mTORC1 and MEK routes while insulin required PI3K), and that these pathways might be common across mammalian species in that they observed similar results using mouse primary pituitary cell cultures (Gahete et al., 2013; Yamashita and Melmed, 1986). Taken together these studies demonstrate IGF-I and insulin can directly regulate somatotrope function under normal conditions (Luque et al., 2006), and since both IGF-I and insulin are regulated by nutritional status, may suggested changes in circulating GH levels observed during starvation or obesity (overeating) may in part be mediated by direct actions of these hormones on somatotrope function.

4.4. Fatty acids

Free fatty acids (FFAs) have also been described as regulators of pituitary function. Specifically, the majority of the information available about the capacity of FFAs to regulate pituitary gland function is related with the modulation of GH secretion. Thus, in primates (rhesus monkeys), it was described a complete inhibition of acute insulin-induced GH secretion after a soybean oil emulsion, which produce an elevation of serum FFAs (Blackard et al., 1969). Consistently, elevation of plasma FFAs produced a strong reduction in GH release in rhesus and Java monkeys and lowering plasma FFAs led to an increase in GH secretion, without altering PRL levels (Quabbe et al., 1990). In healthy humans, as in primates, a reciprocal relationship between FFAs and GH release has been reported (Tsushima et al., 1970; Blackard et al., 1971; Fineberg et al., 1972; Quabbe et al., 1972; Imaki et al., 1985; Casanueva et al., 1987; Maccario et al., 1994; Pombo et al., 1999). Elevations in FFAs induced by different types of lipid infusions are able to mediate a significant inhibition of GHRH-stimulated GH secretion, where it has been hypothesized that this effect is due to suppressing GHRH and/or stimulating SST secretion, or to a direct effect of FFAs on somatotrope cells (Tsushima et al., 1970; Blackard et al., 1971; Fineberg et al., 1972; Quabbe et al., 1972; Imaki et al., 1985; Casanueva et al., 1987; Maccario et al., 1994; Pombo et al., 1999). In support of a direct effect was a report showing that 24 h treatment of baboon primary pituitary cell cultures with oleic and linoleic acids markedly reduced GH release and mRNA levels. In contrast to GH, no association between FFAs concentrations and PRL levels has been observed in primates or humans (Quabbe et al.,

1990; Casanueva et al., 1987). However, like GH, lipid infusion-induced elevations in circulating plasma FFAs evoked a strong inhibitory effect of spontaneous ACTH and cortisol secretion in humans, although the lipid load did not affect CRH-stimulated ACTH levels (Casanueva et al., 1987; Lanfranco et al., 2004). In contrast, another study indicated that FFAs did not alter basal ACTH and cortisol secretion in normal men even when the FFAs levels obtained in response to lipid load were comparable in both studies (Mai et al., 2006). Therefore, further investigations are required to clearly understand the specific role of FFAs at the level of the pituitary gland and the mechanisms involved in such actions.

4.5. Adipokines

Adipokines comprise a family of increasingly important cytokines, mainly released from the adipose tissue, which includes leptin, adiponectin or resistin. However, although certain studies have reported the connection between leptin or adiponectin and pituitary hormones, the precise implication of adipokines on the modulation of human (or primate) anterior pituitary hormones remains to be fully characterized. Indeed, exogenous treatment with leptin in female rhesus monkeys (*Macaca mulatta*) caused a rapid rise in LH concentration, which was followed by an increase in serum estradiol and advanced puberty (Wilson et al., 2003). In addition, leptin was also associated with an elevation of GH secretion in this model (Wilson et al., 2003). Similarly, adiponectin has been directly associated with GH pulse secretion in healthy men, although it remains to be proven whether this is a direct effect (Makimura et al., 2011). Of note, leptin, adiponectin or resistin receptors are expressed in a wide variety of tissues and organs including pituitary gland, wherein they seem to be involved in its regulation (Jin et al., 1999; Psilopanagioti et al., 2009). In order to determine if leptin mediated changes in pituitary hormone secretion is due to direct pituitary actions, a recent report explored the impact of adiponectin, leptin and resistin on primary pituitary cell cultures from two primates species (*Macaca fascicularis* and *Papio anubis*). This study demonstrated that adiponectin reduces GHRH-stimulated but not ghrelin-stimulated GH release, and that it is able to increase PRL and decrease ACTH without altering LH/FSH/TSH-release. Conversely, leptin increased GH, PRL, ACTH and FSH secretion but did not alter LH or TSH secretion. Finally, resistin, like leptin, produced an elevation of GH and ACTH levels without any alteration of PRL, LH, FSH or TSH secretion. In addition, only leptin was able to increase GH, PRL and POMC at mRNA expression levels. Interestingly, the direct effects induced by these adipokines were mediated by common signaling pathways such as AC/PKA, but also involved distinct and specific signaling cascades. Indeed, in addition to AC/PKA, leptin exerted its effects by activating intra-/extracellular calcium and PLC/PKC, adiponectin also involved intra-/extra-cellular calcium, and resistin also induced its effects through mTOR pathway (Sarmento-Cabral et al., 2017). Taken together, these data demonstrate that adipokines could directly modulate the function of anterior pituitary hormones in non-human primates, which could help to explain the results obtained *in vivo* in humans and primates.

4.6. Obestatin

Obestatin is an amidated peptide hormone encoded by the ghrelin gene and mainly produced in the gastrointestinal tract (Zhang et al., 2005; Li et al., 2011). However, the use of human fetal and adult tissue samples has revealed that obestatin is widely distributed throughout human tissue, with prominent expression in lung, pancreas, thyroid, gastrointestinal tract and pituitary gland. Interestingly, a strong correlation between obestatin and ghrelin mRNA levels has been found in these tissues (Volante et al., 2009). The data available in the literature about this hormone is confusing, quite limited and mainly generated in rodent models; however, one study has been recently published exploring the direct, *in vitro*, effect of obestatin on the function of all pituitary cell types using baboon primary pituitary cell cultures as model. Specifically, obestatin treatment did not alter GH or ACTH release or expression after 4 h. However, GH was inhibited, while ACTH/POMC secretion and expression was stimulated, in baboon primary cultures after 24 h of treatment. Additionally, obestatin also blunted ghrelin-stimulated GH release. In contrast, other pituitary hormones (PRL, FSH, LH and TSH) were not affected by obestatin treatment at any time point tested. All these observations suggest that obestatin can directly regulate somatotrope/corticotrope function in primary pituitary cell cultures from baboons, and that these actions are mediated through the activation of AC and MAPK routes (Luque et al., 2014).

4.7. Inhibins

Inhibins are glycoprotein hormones constituted by two different subunits (α- and β_A- or β_B-subunit), which are linked to form inhibin A or inhibin B. These glycoproteins are secreted by the granulosa and theca cells of the ovary and by the Sertoli cells of the testis (Ying, 1988). One of the first evidence demonstrating the effect of inhibins on non-human primate models was published by Medhamurthy et al., where they showed the direct role of inhibins in the regulation of FSH secretion in the male rhesus monkey (*Macaca mulatta*). Specifically, the administration of ovine antiserum against inhibin α-subunit produced a hypersecretion and an increase of pulse amplitude of FSH, but did not alter LH secretion or pattern (Medhamurthy et al., 1990, 1991). In the same line, pituitary FSH secretion and expression were maintained at control values by the infusion of recombinant inhibin in orchidectomized monkeys, preventing the postcastration hypersecretion and overexpression of FSH (Majumdar et al., 1995). In addition, a significant reduction of circulating FSH levels was detected after 54 h when recombinant inhibin was administered by infusion to adult male rhesus monkeys. However, and in line with previous results, the infusion of inhibin A did not alter the circulating LH concentrations in monkeys, which suggests that testicular inhibin actions are specific for FSH at the pituitary level (Ramaswamy et al., 1998). Likewise, exogenous inhibin administration to female rhesus monkeys specifically reduced FSH secretion during the mid-to-late luteal phase of the menstrual cycle (Stouffer et al., 1994). Furthermore, the direct effect of inhibin on FSH and LH secretion in pituitary cell cultures from male rhesus monkeys and one cynomolgus monkey was studied during 48 h of incubation, showing a reduction of 50,8% of FSH release compared with controls while no effect was observed on LH secretion (Fingscheidt et al., 1998). These results were corroborated by another study developed with human fetal primary pituitary cell cultures. In this case, inhibin treatment clearly reduced FSH levels but the effect on LH was inconsistent (Blumenfeld and Ritter, 2001). Regarding the presence and role of inhibins in humans, important gender differences have been described, being inhibin A and B present at physiological concentrations in females, whereas only inhibin B was observed in males (Hayes et al., 1998). In this sense, inhibin B secretion was found to be decreased in older ovulatory women who showed a monotropic FSH increase. On the contrary, inhibin A release in these women was found similar to that in younger women. These results in women suggest that inhibin B has an important role in the modulation of the intercycle FSH changes (Klein et al., 1996). In men, results obtained from an acute sex withdrawal model (declined testosterone and estradiol levels) showed that inhibin B is the

major regulator of FSH release in the human male (Hayes et al., 2001a). Finally, with regard to the action mechanisms, the knowledge about the inhibin effects is quite limited. One of the hypothesis proposed has been that inhibins could act as a dominant negative regulator of the activin signal transduction pathways (see below) through the binding of β_A subunit to the activin type II receptors with lower affinity than activin (Woodruff, 1999). On the other hand, several reports have found non-overlapping binding sites for activin A and inhibin A in different tissues suggesting the existence of inhibin-specific receptors. In this sense, two different candidates has been identified as inhibin receptors, betaglycan (TGF-β type III receptor) and inhibin binding protein/p120 (INHBP/P120). However, none of them seem to satisfy all the criteria required since betaglycan are not expressed in pituitary gonadotrope cells and INHBP/P120 did not bind to inhibins in receptor binding assays (Woodruff, 1999; Bernard et al., 2002). For that reasons, additional studies are necessary to undoubtedly identify inhibins receptor(s) and signaling mechanisms behind the observed effects.

4.8. Activins

Activins, like inhibins, are glycoproteins that belong to TGF-β superfamily. Activins are dimers composed by two different β subunits, which can generate three isoforms: activin A (β_A β_A), activin B (β_B β_B) or activin AB (β_A β_B) (Ying, 1988; Ling et al., 1986). The presence of activins has been detected in some, but not all, Leydig, Sertoli and granulosa cells of fetal primate gonads (Rabinovici et al., 1991). Likewise, β_A subunit was found in FSH-, GH- and in a few PRL-positive cells in human pituitary gland (Uccella et al., 2000). In the same way, β_B subunit was detected in TSH-cells, FSH- and LH-positive gonadotrophs (Uccella et al., 2000). In primates, the first results showing the effect of activins in the function of pituitary gland were obtained from *Macaca fascicularis*. Specifically, 2-days infusion of activin A to adult male macaques produced a significantly increase of basal FSH levels, without changes in basal LH levels. However, GnRH-stimulated FSH and LH levels were significantly increased after 48 h of activin A administration, showing a physiological role of activins on gonadotropin secretion in non-human primates (McLachlan et al., 1989). In the same way, the infusion of exogenous activin to female rhesus monkeys stimulated FSH and LH production during the early follicular phase of the menstrual cycle (Stouffer et al., 1993). On the other hand, the specific direct role of activins on pituitary gland was studied using human fetal primary pituitary cell cultures. In this case, treatment with recombinant activin A produced a potent increase on FSH and LH release, being activin-stimulated LH secretion less potent compared to GnRH treatment (Blumenfeld and Ritter, 2001). To date, the knowledge about the mechanisms and signaling pathways underlying these effects involve the binding of activins to two activin type II receptors (ActRII and ActRIIB), and one type I receptor (ActIR/ALK4). Downstream signaling is mediated by the SMAD signaling pathway, where these SMAD proteins are phosphorylated and translocated to the nucleus as multimeric complexes to regulate gene transcription (Gregory and Kaiser, 2004; Bilezikjian et al., 2012).

4.9. Follistatin

Follistatin (FST), originally called the FSH suppressing protein, is a monomeric polypeptide considered a key regulator of the biological actions of activin. Therefore, this molecule regulates the expression and secretion of gonadotropins contributing to their importance as modulators of the reproductive axis (Welt and Crowley, 1998). FST is secreted from mature gonadal cells, particularly its secretion has been associated to gonadotrophs and folliculostellate cells probably in an autocrine or paracrine manner (Welt and Crowley, 1998). Alternative splicing of this molecule produce two polypeptide variants with different number of amino acids (FST315 and FST288), although with the same mechanisms of action (Shimasaki et al., 1988). The long-variant is distributed throughout the body, while the short-variant is located in secretory tissues (Sugino et al., 1993). Each molecule of FST binds to an activin subunit. The complex activin-follistatin undergoes internalization and lysosomal degradation causing an irreversible activin inhibition, downregulating FSH secretion and avoiding activin-activin receptor binding (Hashimoto et al., 1997). Regarding to the effect of FST in non-human primates, castration of rhesus monkeys produced an increase on FSHβ, LHβ and α-subunit mRNA levels and an increase of FSH secretion, which was related to an unaltered pituitary FST expression in these monkeys (Winters et al., 2001). In humans, a slight decrease of both basal and GnRH-stimulated LH and FSH concentrations in response to FST was detected in human fetal primary pituitary cell cultures, which might be due to the fact that FST could act directly blocking activin actions as it has been described in other species (Blumenfeld and Ritter, 2001).

4.10. Estrogens

There is increasing evidence demonstrating estrogens directly regulate pituitary cell function. In fact, estrogen receptors are expressed in baboon lactotropes and gonadotropes, and to a lesser extent in somatotropes and thyrotropes (Herbert and Sheridan, 1983; Sprangers et al., 1989). The first observations about the relationship between estrogens and GH levels were not conclusive. Specifically, the effect of physiological or pharmacological estradiol doses on the concentration of IGF-I and GH was explored in castrate and intact adult female baboons. These studies demonstrated that only with intact baboons and physiological doses, estradiol was able to increase plasma IGF-I levels, associated with an increase in GH concentrations (Copeland et al., 1984). Likewise, castrated macaques treated with estradiol revealed an increase on GH concentrations. However, estradiol treatment on castrated adult female and male or juvenile female macaques pituitary cell cultures did not show any effect on GH levels, although adult female monkeys showed an increase on PRL secretion. Interestingly, only juvenile male (<2 years), but not adult or juvenile female pituitary cultures presented a mild increase on GH release, and a double immunocytochemistry corroborated a different cell composition between adult and juveniles pituitary cell cultures. Based on these results, the authors suggested that estradiol was acting on a GH-secreting cell population that was present in young male but not in adult monkeys, and that this population was probably composed by mamosomatotrope stem cells, which expressed estrogen receptors (Bethea and Freesh, 1991). In humans, treatment with estradiol decreased IGF-I and elevated basal GH and PRL concentrations in men (Wiedemann et al., 1976). In postmenopausal women, estrogen treatment was able to enhance basal and exercise-induced GH release and decreased IGF-I levels. The mechanisms behind these effects in humans are not clear although possible options could be central effects or a negative feedback related with IGF-I levels (Dawson-Hughes et al., 1986). Regarding the role of estrogens on other pituitary hormones, a direct effect of estradiol and progesterone on PRL secretion has been reported using pituitary cell cultures from male and female monkeys. Thus, estradiol administration significantly increased PRL release compared to vehicle-treated controls. However, estradiol and progesterone combined treatment did not produce any difference in PRL secretion levels compared to estradiol treatment suggesting that progesterone did not exert any effect on PRL secretion (Bethea et al., 1988). In

contrast, PRL levels were not altered in ovariectomized female cynomolgus monkeys treated with estradiol (Sprangers et al., 1990). On the other hand, estradiol has been described as the predominant regulator of FSH secretion in men through the aromatization of testosterone to estradiol (Hayes et al., 2001b). Taken together, further studies are necessary to clearly elucidate the role estrogens play on anterior pituitary hormones and the signaling pathways underlying these effects.

4.11. Testosterone

In human and monkeys, testosterone acts as a gonadal component of the negative feedback that regulate LH and FSH secretion; however, the precise actions of testosterone on gonadotropin secretion in humans and non-human primates seem not to be the same (Fingscheidt et al., 1998; Matsumoto and Bremner, 1984). In this sense, it has been demonstrated that testosterone replacement after orchidectomy failed to prevent the postcastration FSH hypersecretion in male rhesus monkeys without altering LH levels, which suggests that circulating testosterone concentrations are not essential for the testicular inhibition of FSH secretion in rhesus monkeys (Dubey et al., 1987). In the same line, treatment with testosterone did not produce any change on basal or GnRH-stimulated FSH or LH levels in primate pituitary cell cultures (Fingscheidt et al., 1998; Kawakami and Winters, 1999). In contrast to these data, the results reported in humans reveal that testosterone or its metabolites are able to inhibit FSH and LH secretion acting at the pituitary and hypothalamus level. Moreover, although the effect of testosterone on LH release appears to be through a direct or indirect feedback, the aromatization of testosterone to estradiol seem to be necessary to produce an effect on FSH secretion (Hayes et al., 2001b; Matsumoto and Bremner, 1984; Sheckter et al., 1989; Finkelstein et al., 1991). However, the signaling pathways associated to these effects have not been described. For these reasons, further investigations are necessary to clarify the effects of testosterone on gonadotropin hormones and the mechanisms underlying these effects.

4.12. Endothelin

Endothelin (ET) is a peptide that contributes to constrict the blood vessel and to rise blood pressure and, consequently, overexpression of this molecule is associated with heart diseases. In human, three different ET isopeptides encoded by three different genes were identified and designated as ET-1, ET-2 and ET-3 (Inoue et al., 1989). The presence of ET-3 in gonadotrophs cells has been detected using immunoreactivity suggesting a potential role of ETs in gonadotropins secretion (Naruse et al., 1992; Takahashi et al., 1991; Lange et al., 1994). *In vivo* assays with healthy human male volunteers showed that ET-1 intravenous administration produced an increase on basal serum ACTH levels without altering the rest of pituitary hormones. However, the increase of pituitary hormones secretion stimulated by pituitary hormones releasing factors (GHRH, CRH, GnRH, TRH) was altered in some cases after ET-1 administration. Thus, TSH-stimulated PRL levels and GHRH-stimulated GH levels were decreased after ET-1 administration. In contrast, ACTH, FSH and LH were enhanced and TSH was unaltered in response to ET-1 treatment (Vierhapper et al., 1993). In a different study, the effect of ET-1 and ET-3 administration was further studied in men. In this sense, ET-1, but not ET-3, increased plasma ACTH and PRL levels (Kiefer et al., 2000). Regarding the mechanisms involved in ET actions, it is known that ACTH and GH concentrations decreased when nifepidine (a calcium channel blocker) was administered before ET-1 infusion, without any alteration on other pituitary hormones. Based on these data, it has been suggested that the effect observed in human in response to ET could be, at least in part, mediated by calcium mobilization at the pituitary level (Vierhapper, 1996).

4.13. Opioids

Opioids encompass any endogenous or exogenous agent that binds to opioid receptors, which are located mainly in the central nervous system. A significant amount of reports have identified the main types of receptors as mu-μ, kappa-κ and delta-δ opioid receptors (Sadee et al., 1982). The effect of opioids at the pituitary level depends on the cell type implicated. For instance, intrathecal administration of opioids was able to modulate different pituitary hormones in a group of 73 patients. The consequence of the chronic and acute administration was a significant decrease on serum LH concentrations and, only in the chronic administration, FSH levels (Abs et al., 2000; Delitala et al., 1983; Pende et al., 1986) through the μ-opioid receptor pathway (Mauras et al., 1987). The effect observed on LH release was dependent on the sexual maturation stage of patients due to the sex steroid hormones, which are required for major modulating effects (Petraglia et al., 1986; Kletter et al., 1991, 1997). The effect of chronic opioid administration on PRL levels is not clear since the information in the literature is contradictory. Likewise, PRL levels were not modified in chronic patients (males and females) that received opioids either intrathecally or orally (Abs et al., 2000; Fraser et al., 2009). However, acute dose of morphine caused an increase on PRL levels, demonstrating that this effect is achieved through dopaminergic mechanisms (Delitala et al., 1983). In this sense, in non-human primates, PRL release was enhanced by dopaminergic pathways (Wehrenberg et al., 1981). The use of opioid antagonists showed an increase of LH levels that could be caused by a change on GnRH levels (Tenhola et al., 2012). On the other hand, opioids increased plasma GH level through a reduction of somatostatin tone in healthy males. This conclusion was obtained after two studies using naloxone administration (Tomasi et al., 1998; Barbarino et al., 1987). In addition, TSH was elevated after opioids administration as it is demonstrated in different studies (Delitala et al., 1983; Pende et al., 1986; Roti et al., 1984). Specifically, the use of opioids and their antagonists had greater effects in modifying the nocturnal pulses of TSH by altering the circadian rhythm of this hormone (Samuels et al., 1994; Leslie et al., 1985). However, these results regarding TSH levels were not corroborated by another study (Abs et al., 2000). Regarding ACTH levels, several reports indicated that the use of these compounds reduced the pituitary ACTH response to CRH through κ-receptor (Allolio et al., 1987; Naber et al., 1981; Geer et al., 2005; Palm et al., 1997; Conaglen et al., 1985; Rittmaster et al., 1985; Grossman et al., 1986; Pfeiffer et al., 1986). Altogether, the opiods seem to have a direct role at the hypophyseal level in humans. Nevertheless, the information found in the literature is contradictory in many cases, which suggests that additional studies are necessary to clarify the real effect on pituitary hormones and the mechanisms involved in these effects.

5. Signaling pathways involved in the regulation of pituitary gland

As reviewed in detail above, the vast majority of the information and knowledge regarding the signaling pathways involved in the regulation of the synthesis and release of the different anterior pituitary hormones has been generated using primary pituitary cell cultures from non-human primate species. Indeed, almost all the studies referenced in this review report the effect of the different regulators on hormone release; however, not all of them explored the effect on hormone expression. For this reason, it would be

necessary to further explore this particular question in order to better understand the differential regulation of pituitary hormonal synthesis and release by these regulators. On the other hand, the major findings regarding signaling pathways of all studies included in this review are summarized in Table 1. In particular, in these studies, the main approach used to explore the signaling pathways activated or inhibited in response to different pituitary regulators has been the direct measurement of key second messengers coupled to the use of specific pharmacological inhibitors to block selected components of relevant routes. An overall view of all the information available reveals that most of the data reported hitherto in the literature is mainly focused in the mechanisms involved in the regulation of GH release by different central and peripheral modulators (Table 1). When taken together, these data indicate that the regulation of GH release by different modulators is carried out through the modulation of multiple, common and distinct, signaling pathways. Specifically, most of the GH regulators act through two common signaling pathways such as AC/PKA (except for kisspeptins) (Kineman and Luque, 2007; Ibáñez-Costa et al., 2015; Gahete et al., 2009; Córdoba-Chacón et al., 2012; Mayo et al., 2000; Córdoba-Chacón et al., 2011; Murakami et al., 2001; Sarmento-Cabral et al., 2017) and extra- and/or intracellular calcium mobilization (except for PACAP, resistin and obestatin) (Kineman and Luque, 2007; Ibáñez-Costa et al., 2015; Córdoba-Chacón et al, 2011, 2012; Luque et al., 2011; Sarmento-Cabral et al., 2017; Vierhapper, 1996). In addition, most of the modulators of GH secretion simultaneously elicit the activation and/or inhibition of additional routes. Indeed, ghrelin and kisspeptins modulate GH release also through PLC/PKC and MAPK pathways (Kineman and Luque, 2007; Luque et al., 2011), while MT also regulate PLC/PKC pathway (Ibáñez-Costa et al., 2015). Alternatively, GHRH-mediated GH release required NOS/NO/GC/cGMP pathway (Kineman and Luque, 2007), obestatin is also able to inhibit GH release through MAPK signaling pathways (Luque et al., 2014), and adipokines use PI3K,whereas resistin activates the mTOR pathway to regulate GH release (Sarmento-Cabral et al., 2017).

Regarding PRL regulation, MT, leptin and adiponectin are able to exert their effects on PRL secretion through AC/PKA pathway and extra-/intra-cellular Ca^{2+} mobilization (Ibáñez-Costa et al., 2015; Sarmento-Cabral et al., 2017). The stimulation of PRL release by leptin and adiponectin also involves the activation of PI3K pathway (Sarmento-Cabral et al., 2017). On the other hand, the regulation of ACTH is mediated through AC/PKA by ghrelin, obestatin and adipokines, through MAPK by ghrelin and obestatin, through PI3K by adipokines (Kineman and Luque, 2007; Luque et al., 2014; Sarmento-Cabral et al., 2017), and also through extracellular Ca^{2+} mobilization by endothelins (Vierhapper, 1996). In the case of gonadotropins, both LH and FSH hormones are differentially regulated by distinct but also by some common signaling pathways. Likewise, LH is modulated through PLC/PKC, intracellular Ca^{2+} mobilization, MAPK, mTOR and PI3K by kisspeptins, through extracellular Ca^{2+} mobilization and NOS/NO/GC/cGMP pathway by GnRH (Luque et al., 2011), through intracellular Ca^{2+} mobilization and AC/PKA by GnIH (Clarke and Parkington, 2014; Son et al., 2012) and through SMAD signaling by activins (Gregory and Kaiser, 2004; Bilezikjian et al., 2012). Additionally, FSH release mediated by GnIH

Table 1
Summary of the signaling pathways modulated by different regulators on the secretion of anterior pituitary hormones.

Hormone	Signaling pathways	Regulators	References
GH	AC/cAMP	GHRH, Ghrelin, CORT, SST, PACAP, MT, Leptin, Adiponectin, Resistin, Obestatin	(Kineman and Luque, 2007; Luque et al., 2014; Ibáñez-Costa et al., 2015; Gahete et al., 2009; Córdoba-Chacón et al., 2012; Mayo et al., 2000; Córdoba-Chacón et al., 2011; Murakami et al., 2001; Sarmento-Cabral et al., 2017)
	Extra- and/or intracellular Ca^{2+} mobilization	GHRH, Ghrelin, CORT, SST, MT, Kisspeptins, Leptin, Adiponectin, Endothelin	(Kineman and Luque, 2007; Ibáñez-Costa et al., 2015; Córdoba-Chacón et al., 2012; Córdoba-Chacón et al., 2011; Luque et al., 2011; Sarmento-Cabral et al., 2017; Vierhapper, 1996)
	PLC/PKC	GHRH, Ghrelin, MT, Kisspeptins	(Kineman and Luque, 2007; Ibáñez-Costa et al., 2015; Gracia-Navarro et al., 2002; Luque et al., 2011)
	PI3K	Leptin, Adiponectin, Resistin	(Sarmento-Cabral et al., 2017)
	MAPK	Ghrelin, Kisspeptins, Obestatin	(Kineman and Luque, 2007; Luque et al., 2014; Luque et al., 2011)
	mTOR	Resistin	(Sarmento-Cabral et al., 2017)
	NOS/GC	GHRH	(Kineman and Luque, 2007)
PRL	AC/cAMP	MT, Leptin, Adiponectin	(Ibáñez-Costa et al., 2015; Sarmento-Cabral et al., 2017)
	Extra- and/or intracellular Ca^{2+} mobilization	MT, Leptin, Adiponectin	(Kineman and Luque, 2007; Sarmento-Cabral et al., 2017)
	PI3K	Leptin, Adiponectin	(Sarmento-Cabral et al., 2017)
ACTH	AC/cAMP	Ghrelin, Obestatin, Leptin, Adiponectin, Resistin	(Luque et al., 2014; Camiña et al., 2007; Coiro et al., 2005; Sarmento-Cabral et al., 2017)
	MAPK	Ghrelin, Obestatin	(Luque et al., 2014; Mousseaux et al., 2006; Camiña et al., 2007)
	PI3K	Leptin, Adiponectin, Resistin	(Sarmento-Cabral et al., 2017)
	Extra-cellular Ca^{2+} mobilization	Endothelin	(Vierhapper, 1996)
LH	Extra- and/or intracellular Ca^{2+} mobilization	GnRH, Kisspeptins, GnIH	(Luque et al., 2011; Clarke and Parkington, 2014; Son et al., 2012)
	PLC/PKC	Kisspeptins	(Luque et al., 2011)
	MAPK	Kisspeptins	(Luque et al., 2011)
	mTOR	Kisspeptins	(Luque et al., 2011)
	PI3K	Kisspeptins	(Luque et al., 2011)
	NOS/GC	GnRH	(Luque et al., 2011)
	SMAD	Activins	(Gregory and Kaiser, 2004; Bilezikjian et al., 2012)
FSH	AC/cAMP	GnIH, Leptin	(Son et al., 2012; Sarmento-Cabral et al., 2017)
	PLC/PKC	Leptin	(Sarmento-Cabral et al., 2017)
	PI3K	Leptin	(Sarmento-Cabral et al., 2017)
	Extra- and intra-cellular Ca^{2+} mobilization	GnIH, Leptin	(Clarke and Parkington, 2014; Son et al., 2012; Sarmento-Cabral et al., 2017)
	SMAD	Activins	(Gregory and Kaiser, 2004; Bilezikjian et al., 2012)

involve intracellular Ca^{2+} mobilization and AC/PKA (Clarke and Parkington, 2014; Son et al., 2012), by leptin involve AC/PKA, PLC/PKC, extra-/intra-cellular Ca^{2+} mobilization and PI3K (Sarmento-Cabral et al., 2017) and by activins also involve SMAD signaling (Gregory and Kaiser, 2004; Bilezikjian et al., 2012). Taken together, all this information suggests that the central and peripheral modulators mentioned above, in most cases, converge in multiple and similar signaling pathways to regulate the function of different anterior pituitary cell types (Table 1). However, only some selected signaling pathways have been explored in these studies, which suggest that more *in vitro* studies are necessary to understand the full landscape of signaling pathways involved in the regulation of pituitary gland function in humans and in non-human primate models.

6. Concluding remarks

This review summarizes what we know to date regarding both central and peripheral factors involved in the regulation of pituitary cell function (Fig. 1), specially focusing on studies performed in humans and non-human primates, and paying special attention to intracellular mechanisms underlying this regulation. Although some regulators seem to exert discrepant results depending on the study, it seems solidly demonstrated that the regulation of pituitary function is triggered by an integration of multiple factors acting simultaneously and/or sequentially at this gland, which converge, and ultimately result, in the activation and/or inhibition of multiple, common and distinct, signaling pathways to finely modulate the synthesis and secretion of the different anterior pituitary hormones. The broad perspective gained through this review highlight the importance of the pituitary gland, often referred to as the "master endocrine gland" of the organism, as a true sensor of whole body function, able to gauge the status of growth, reproduction, lactation, stress, metabolism and in turn adjust pituitary hormone synthesis and release to finely control the whole-body homeostasis. This growing number of regulators, interactions and mechanisms, supports the view that the control of pituitary function is far more complex than originally envisioned, and that future studies will need to be implemented in order to elucidate the precise effects of various regulators mentioned in this review, the complete set of their underlying mechanisms, and the network of interactions among them.

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